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The Effects of Poor Maternal Nutrition on the Growth, Development, and Metabolism of Offspring

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Abstract

Poor maternal nutrition during gestation can lead to intrauterine growth restriction resulting negative impacts on the growth development and metabolism of the offspring. Specifically, poor maternal nutrition during gestation will lead to a reduction in muscle and bone content, increase adiposity and alter organ development in offspring. Although studies have elucidated the impacts of poor maternal nutrition on multiple tissues at different time points in the life of offspring, the potential mechanisms behind these alterations need to be studied in detail. We hypothesized that maternal under- and over-nutrition during gestation in sheep will 1) alter the development of muscle, bone, adipose and vital organs in offspring 2) impact the proliferation, differentiation, and metabolism of mesenchymal stem cells (MSC) and 3) alter the expression of key genes and novel pathways involved in the prenatal muscle. To test these hypotheses, two independent in-vivo experiments were conducted using sheep as a model. In the first study, thirty-six pregnant ewes were individually housed and randomly assigned to one of three diets (100%, 60%, or 140% of National Research Council (NRC) requirements for TDN) at d 31 ± 1.3 of gestation. Lambs from these ewes were euthanized within 24 hours of birth and 3 months of age to obtain samples to conduct further studies. Maternal diet negatively affected offspring MSC by reducing proliferation 50 % and reducing mitochondrial metabolic activity ($P \leq 0.04$). In the second in-vivo study, eighty-two pregnant ewes were individually housed and randomly assigned to one of three diets (100%, 60%, or 140% of NRC requirements for TDN) at d 30 ± 0.2 of gestation. Lambs from these ewes were euthanized at different time points of gestation and at birth to obtain the samples to conduct further studies. Although no significant

interactions of diet by day of gestation and main effect of poor maternal nutrition were observed for fiber CSA of Longissimus muscle, semitendinosus (STN), and triceps brachii (TB) ($P \geq 0.28$), there was a significant main effect of time on the fiber CSA of LM, STN, and TB ($P \leq 0.0001$). Samples from longissimus dorsi were used to perform transcriptome analysis to evaluate the differential gene expression. Data from transcriptome analysis found alterations in the expression of genes involved in epigenetic regulation and cell signaling as a result of exposure to poor maternal nutrition ($q \leq 0.05$). In conclusion, poor maternal nutrition affects the growth and development of organs, adipose and muscles through different mechanisms. The potential mechanisms that were identified as a part of these studies were alterations in the gene expression and stem cell functions. Future studies are needed to identify the role of epigenetic modifications as a potential mechanism behind the observed impacts of poor maternal nutrition in offspring.

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Doctor of Philosophy Dissertation

The Effects of Poor Maternal Nutrition on the Growth, Development, and Metabolism of Offspring

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DEDICATION

This dissertation is dedicated to my grandmother Shantha Kumary, my parents Muraleedharan Pillai and Geetha Kumary, my wife Mithra Pillai, my brother Sabarinadh and son Shivshankar

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REVIEW OF LITERATURE

Introduction

Agriculture contributes 394.6 billion dollars in revenue each year, and 182.2 billion dollars of this income is contributed by the livestock sector (USDA, NASS Census, 2012). In the United States, 50% of total farm revenue and 40% of farm income are generated from the sale of animals for meat (Gerrard and Grant, 2006). Meat and meat products are a staple in US diets and contribute to 40% of daily protein intake and 20% of fat intake (Daniel et al., 2011). Consequently, the US livestock industry needs to meet the demands for high-quality meat products for an ever-increasing global population. Therefore, animals with reduced productivity and overall general health are a concern for the producer and consumer due to decreased profitability and food production by reducing the quality and quantity of meat produced. Several genetic and environmental factors and their interactions can affect the overall growth, development, and health of animals (Godfrey and Barker, 2001; Wu et al., 2006; Simmons, 2011). Some of these factors such as stress and maternal diet can affect offspring before birth, within the maternal environment, in a process known as fetal programming (Yates et al., 2012). Fetal programming is defined as the process by which a stimulus or insult at a critical, sensitive period of early life has permanent effects on structure, physiology, and metabolism in the remaining life of the offspring (Godfrey and Barker, 2001). Fetal programming can be caused by factors such as uterine capacity, placental vasculature, maternal nutrition and pathological conditions caused by metabolic and infectious diseases (King, 2003a; Wu et al., 2006).

In-utero, offspring growth and development are closely associated with maternal nutrition during gestation. Poor maternal diet can program the fetus and may have lifelong consequences

(Nesterenko and Aly, 2009; Simmons, 2011; Yates et al., 2012; Varadinova et al., 2015). The focus of research discussed in this dissertation will be on poor maternal nutrition and its impacts on growth and development of offspring. Partitioning of nutrients to tissues during in-utero development follows a hierarchy. When the availability of nutrients is altered, as in the case of under- or over-nutrition during gestation, tissues that receive a low priority during development, such as muscle, are impacted to a greater extent in comparison with brain or heart (Du et al., 2010a). In addition to the changes observed in the tissues, poor maternal nutrition also affects the multipotent stem cells that are key to the development of different tissues in-utero and maintenance of these tissues postnatally. The hierarchy of partitioning is also evident in the case of different organ systems. Therefore, the tissues and organs are impacted to varying degree depending on their hierarchical order during the partitioning of nutrients.

Early origin of adult disease and fetal programming

In 1997, Barker proposed that in utero growth restriction, as manifested by low birth weight (LBW), increases the risk of cardiovascular disease and stroke (Barker, 1997). Later, Barker and Hales, provided a mechanistic explanation to the impacts of poor maternal nutrition by proposing the thrifty phenotype hypothesis (Barker, 1998; Barker and Hales, 2001; Hales and Barker, 2013). The thrifty phenotype hypothesis proposed that adaptations during critical phases of growth and development may ensure the maintenance of homeostasis, and hence survival, when the environment is compromised (Godfrey and Barker, 2001; Barker and Hales, 2001). It was also proposed that variation in the maternal nutrient intake during early developmental processes might be a strong signal which initiates the predictive adaptive responses (Godfrey and Barker, 2001; Barker and Hales, 2001). Fetal programming is the general idea that important physiological parameters can be reset by environmental events during development of the

embryo and fetus (Godfrey and Barker, 2001). It is to be noted that these changes can endure into adulthood and can be transmitted across generations (McMillen and Robinson, 2005; Langley-Evans, 2006; Godfrey et al., 2010).

Programming is the result of the innate ability of the developing tissues to adapt to the existing conditions during early life. This adaptation might be beneficial in short-term but may lead to consequences in long-term survivability and health of the individual (Reynolds et al., 2010; Yates et al., 2012). For instance, if the postnatal conditions of the programmed individuals are optimal and nutrients are abundant, the organism is ill-prepared to cope with the different environment and hence is more susceptible to developing diseases (McMillen and Robinson, 2005; Godfrey et al., 2010; Costello et al., 2013; Meyer-Gesch et al., 2013). Recent studies on fetal programming have revealed that the effects of programming can be permanent and are transmitted across generations (Simmons, 2011; Lee, 2015). Although the mechanisms behind these effects are still being evaluated, epigenetic mechanisms are now known to play a key role in the observed changes.

Poor maternal nutrition

Poor material nutrition is any alteration to micro- or macro-nutrient ingestion during gestation and can lead to immediate and long-term detrimental effects on the overall growth and development of offspring (Wu et al., 2006). Poor maternal nutrition can be caused by ingestion of nutrients in limited or excess quantity, or ingestion of diets that contains inadequate or excess specific nutrients (Kind et al., 2005; Wu et al., 2006; Ford and Long, 2011). Two examples of poor maternal nutrition encountered most commonly in livestock are under- and over-feeding (Fiorotto et al., 1995; Ford and Long, 2011). Low availability and quality of feed arising as a

result of seasonal variation, harsh climatic condition or insufficient feed production, and bad management practices can result in under-nutrition (Wu et al., 2006). Management practices such as flushing, and access to ad-libitum feed or grazing in rich pasture can lead to over-nutrition (Jackson et al., 2012). It is understood that both under- and over-nutrition can affect offspring development through multiple mechanisms (Wu et al., 2006; Almond and Currie, 2011; Ford and Long, 2011; Hoffman et al., 2016b).

Both under- and over-nutrition increase neonatal morbidity and mortality in addition to altering post-natal growth (King, 2003b; Redmer et al., 2004; Wu et al., 2006). Evidence derived from multiple studies evaluating under- or over-nutrition suggests that exposure to poor maternal nutrition detrimentally affects the prenatal and postnatal growth trajectory of eutherian mammals (Osgerby et al., 2002; Han et al., 2004; Wu et al., 2006; George et al., 2010; Ford and Long, 2011; Ge et al., 2013). Critical windows of development are important periods in the course of development during which specific tissues are particularly sensitive to the influences of environment (McMillen and Robinson, 2005; Reynolds et al., 2010; Simmons, 2011). Any insults to the tissues during these phases of growth can adversely and irreversibly impact the development of the specific tissue (Wu et al., 2006; Simmons, 2011). Another concept that is associated with poor maternal nutrition is the concept of compensatory or catch-up growth. This type of growth is defined by an accelerated phase of growth following a period of slow development during exposure to poor maternal diet (Douglas-Denton et al., 2002; Kind et al., 2005; Tarry-Adkins et al., 2013). For instance, in-utero, maternal nutrient restriction can slow the growth of offspring during gestation and may lead to reduced birthweight (Kind et al., 2005; Wu et al., 2006; Hoffman et al., 2014). These animals may recover and gain the same body weight as control animals during their postnatal life through catch-up growth (Tarry-Adkins et al., 2013).

Although the overall body weight is compensated, the tissue specific growth might be altered leading to changes in body composition, such as increased adipose tissue development (Fiorotto et al., 1995; Kind et al., 2005). These changes in specific tissue development during prenatal development can later lead to negative consequences on the overall development and metabolism in the adult life of organism (Nesterenko and Aly, 2009; Ware et al., 2015; Varadinova et al., 2015).

Normal organ development and function

Adequate development of individual organs is essential for the proper function of interdependent organ systems (Reynolds et al., 2010; Luyckx and Brenner, 2015). Subsequently, the proper function of the interdependent organ systems is important for maintaining general health and homeostasis in young and adult animals (Wu et al., 2006; Simmons, 2011). Normal activity of vital organs such as liver, heart, and kidney are important for the immediate and long-term survival and well-being of the animals (Hoet and Hanson, 1999; Kmiec, 2001; Douglas-Denton et al., 2002; Hyatt et al., 2008). Liver is the largest gland in the body exhibiting both endocrine and exocrine properties (Hyatt et al., 2008). Bile secretion is the major exocrine function whereas Insulin-like growth factors (IGF), Insulin-like growth factor binding proteins (IGFBPs), angiotensinogen, and thrombopoietin are the major endocrine secretions of the liver (Georgieva et al., 2003). Liver also plays a central role in regulating glycogen storage, drug detoxification, control of metabolism, regulation of cholesterol synthesis and transport, urea metabolism, and secretion of an extensive array of plasma proteins (Argiles et al., 2001; Liu et al., 2013). Liver is an organ which develops during early gestation and liver organogenesis is an excellent example of the stepwise developmental process (Kmiec, 2001; Hyatt et al., 2008). First, the endothelial cells differentiate to form embryonic foregut and acquire the capacity to express

hepatocyte-specific genes (Douart et al., 2015). Subsequently, immature hepatocytes differentiate into mature hepatocytes and intrahepatic bile ducts (Douart et al., 2015). These developmental processes are regulated by intrinsically programmed mechanisms and extracellular signals which modulate liver cells to either multiply, differentiate or undergo apoptosis (Kmiec, 2001; Hyatt et al., 2008). Also, these signals are sensitive to the environment encountered during the early organogenesis of liver (Vonnahme et al., 2003; Hyatt et al., 2007; Hyatt et al., 2008; McCurdy et al., 2009).

The primary function of heart is to pump oxygenated blood to different parts of the body and deoxygenated blood to the lungs via the pulmonary artery. The primordial structures of the heart develop from the mesodermal tissues during the process of gastrulation (Burrell et al., 2003; Krishnan et al., 2014). This occurs around the third week of development in humans and 7 days after fertilization in mouse (Krishnan et al., 2014). During fetal development, the heart is enlarged by an increase in both the number (hyperplasia) and size (hypertrophy) of cardiac myocytes (Burrell et al., 2003). Fetal heart development occurs by hyperplasia and binucleation of cardiomyocytes and hypertrophic growth of cardiomyocytes (Field, 1946; Burrell et al., 2003). At birth, the human heart contains almost the full set of cardiomyocytes that it will have for life (Field, 1946; Krishnan et al., 2014). Development of human and sheep heart is similar in that binucleation of cardiomyocytes also occurs predominantly before birth, with the hyperplastic growth of the cardiomyocytes occurring before 110 days of gestation followed by a transition to hypertrophic growth by term in sheep (Krishnan et al., 2014). In sheep, at 135 days of gestation, the proportion of cardiac myocytes that are binucleated is 50%, with nearly 80% being binucleated at 145 to 146 days of gestation (Burrell et al., 2003). This suggests that the majority of the hyperplasia of cardiomyocytes is completed in sheep before birth (Field, 1946; Burrell et

al., 2003). Also, fetal circulating growth factors, angiotensin, and cortisol are known to impact the hypertrophic growth in fetal sheep (Nishina et al., 2003; Le Clair et al., 2009). Therefore, alterations to the amount of these factors in circulation can impact the development of heart (Morrison et al., 2006). It is clear that prenatal period is a key phase of cardiovascular development and in-utero environment influence development of heart. Therefore, adverse environment during prenatal development can lead to the alterations in the functions of offspring heart, which can subsequently affect the functioning of offspring in its postnatal life.

The most important functions of the kidney are waste excretion and maintaining water balance in the body. In addition, they are important for blood pressure regulation, red blood cell regulation, and maintaining acid-base balance in the body. Kidney tissue also arises from the intermediate mesoderm in three different phases (Moritz and Wintour, 1999). The first stage is the development of pronephros which are marked by nephrotomes and are considered non-functional in mammals (Moritz and Wintour, 1999). The second stage involves the development of mesonephros, and the third stage is characterized by the development of metanephros and maturation of metanephros into functional kidney (Moritz and Wintour, 1999; Woods et al., 2001). Kidney is a vital oorgan which starts developing from very early stage of gestation in sheep (Moritz and Wintour, 1999; Woods et al., 2001). In sheep, first-trimester kidney was found to have numerous mesenchymal cells and connective tissues with only few developing glomerulus (Bello et al., 2016). The process of metanephrogensis in sheep starts around 30 days of gestation and continues up to day 135 of gestation (Moritz and Wintour, 1999). Few developing glomeruli and immature developing duct system are found in the second trimester whereas numerous developed glomeruli and connective tissue are seen in the third trimester (Bello et al., 2016). The maturation and development of ovine kidney is completed during the

early post-natal development. Kidney development in sheep is a process that spans the entire gestation and therefore can be influenced by changes in intrauterine environment at various stages of gestation.

Development of muscle

Function and significance of muscles

Animal carcasses are composed of muscle, fat, bone and connective tissue, of which skeletal muscle is the most important (Du et al., 2010b) because it contributes the most to the meat produced. Muscle tissue comprises 40 to 75% percent of the body mass and serves a variety of functions in the body such as aiding in locomotion and regulating metabolism. The contractions and relaxation of skeletal muscle help to generate force and power, maintain posture, and produce movement that influences activity, maintains or enhances health, and contributes to functional independence. Also, skeletal muscles develop special functions as in sphincter muscles where they regulate the movement of body fluids and materials within the body (Frontera and Ochala, 2015). In addition to the functions associated with the mechanical force generated by skeletal muscles, skeletal muscles are necessary for some homeostatic functions involved in regulating metabolism. This includes contributions to basal energy metabolism, serving as storage for important substrates such as amino acids and carbohydrates (Frontera and Ochala, 2015). Shivering thermogenesis and glucose concentration maintenance are examples of critical homeostatic functions of skeletal muscles which are essential for the survival of organisms (Meyer et al., 2002). Muscle is also the major component of protein source in meat and therefore optimum muscle development is essential for profitable meat production (Tygesen et al., 2007; Du et al., 2013). Fiber composition, collagen content, intramuscular

adipose content, cross-sectional area, and fiber density of muscles are factors that can affect the function as well as the quality and quantity of meat produced (Oksbjerg et al., 2004). Therefore, there is a need to understand and discuss the composition of muscle, physiology of muscle function and of the process of muscle development (myogenesis) in detail.

Structure of muscle

The building units of muscles are muscle fibers and therefore the size of a muscle is determined by the number and size of individual muscle fibers. Muscle fibers are post-mitotic and multinucleated. This is important because the formation of fibers are limited at birth and myonuclear domain plays a huge role in the development and function of muscle fibers. Each individual muscle fiber is covered by a sarcolemma, and a group of muscle fibers are surrounded by a layer of connective tissue called perimysium (Frontera and Ochala, 2015). The muscle as a whole is surrounded by connective tissue called epimysium (Oksbjerg et al., 2004). Each muscle fiber is composed of contractile, regulatory, and cytoskeletal proteins (Frontera and Ochala, 2015). The individual muscle fibers are made up of thousands of myofibrils which are, in turn, composed of myofilaments (Bentzinger et al., 2012). Actin and myosin are the two most abundant myofilaments and comprise 70 to 80% of total protein content of muscle (Bentzinger et al., 2012). A total of eleven sarcomeric myosins have been identified in mammals, and these are the molecular motors of myofilaments (Bentzinger et al., 2012; Frontera and Ochala, 2015). Other proteins that are involved in the excitation and contraction processes are the troponin complex and tropomyosin that regulate the sliding action of the actin filament (Frontera and Ochala, 2015). Titin and nebulin are other proteins involved in the regulation of contractile properties, integrity of the sarcomere, influence passive tension and stiffness, and may be relevant to the assembly of myofibrils and cell signalling (Frontera and Ochala, 2015).

The muscle fibers can be classified according to their biochemical, mechanical, and metabolic phenotypes. The predominance of the type of muscle fibers present in a muscle vary depending on the functions of the muscles (Daniel et al., 2007). different fiber types, whose identity is first established during embryonic development by intrinsic myogenic control mechanisms and is later modulated by neural and hormonal factors. The three fiber types are type 1, type 2A and type 2B fibers. Type 1 are the slow, oxidative, fatigue-resistant fibers, type 2A which are the fast-twitch oxidative glycolytic and type 2B which are the fast, glycolytic and fatigable muscle fibers (Schiaffino and Reggiani, 2011). Recent studies in mice have also pointed out the existence of a fourth muscle fiber type known as type 2X, which is intermediate between type 2A an type 2B (Schiaffino and Reggiani, 2011). Muscle fibers suited to each different task have been developed as a part of evolution and this has contributed to the current heterogeneity that is seen in higher order animals. Connection of the various fiber types with motor neurons and the nature of their activity is key for the functional properties of each fiber and fiber type remodelling throughout development (Bentzinger et al., 2012; Confortim et al., 2015; Frontera and Ochala, 2015). Fiber type diversification may also reflect an adaptation to whole body metabolism, and is especially sensitive to changes in glucose and protein metabolism which can be caused by extrinsic factors (Oksbjerg et al., 2004; Schiaffino and Reggiani, 2011). Type of myosin heavy chain is associated with the muscle fiber types and the presence and predominance of specific myosin heavy chain are key feature of specific fiber types (Schiaffino and Reggiani, 2011; Bentzinger et al., 2012; Frontera and Ochala, 2015). Muscle function, development and metabolism is a tightly regulated by multiple factors and is key for overall development and function of the organism.

Myogenesis

Myogenesis is a classical example of tightly regulated progenitor cell maintenance, lineage specification, and terminal differentiation. The process of myogenesis is divided into several distinct phases and is tightly regulated by an interplay of intrinsic and extrinsic regulatory mechanisms (Bentzinger et al., 2012). Skeletal muscles cells of eutherian mammals arise during mid-gestation from three different locations such as the segmented somatic paraxial mesoderm, the unsegmented cranial paraxial mesoderm and the prechordal mesoderm. During embryogenesis, first, muscle fibers are generated from mesoderm-derived progenitor cells in a process called primary myogenesis (Bentzinger et al., 2012). Spatiotemporal somitogenesis is the development of the somites from the mesoderm, and this involves the expression of the Notch and Wingless and INT-1 proteins (Wnt) pathways (Francis-West et al., 2003). The dorsal portion of the somites develop into the dermomyotome and majority of skeletal muscle are derived from the cells of dermomyotome (Francis-West et al., 2003). These cells are characterized by the expression of *Pax3* and *Pax7* and low expression myogenic transcription factor *Myf5* (Kiefer and Hauschka 2001). The myotome develops from the dermomyotome, and the cells of dermomyotome express high levels of myoblast determination protein (*MyoD*) and *Myf5* which are markers of committed muscle cells and are terminal markers of the specification to muscle lineage (Pownall et al. 2002).

In a subsequent wave of myogenesis, new fibers are formed using the primary myofibers as a template. The initial rapid proliferation of the progenitor cells decreases and is accompanied by a spike in protein synthesis and hypertrophy of an individual muscle fiber. After the maturation of muscles, the progenitor cells will reside within as satellite cells. Postnatal muscle development and maintenance depend on the hypertrophy of the existing muscle fibers and activation of satellite cells to differentiate into new fibers. It is important to note that the process

of hyperplasia, or formation of new muscle fibers, does not happen in large mamamals after birth which also means that the number of muscle fibers are determined by birth. Therefore, the period of in-utero growth is a critical period for myogenesis in eutherian mammals.

Regulation of myogenesis

Many specific signaling molecules regulate and direct the process of prenatal and postnatal myogenesis. These factors are responsible for the translation of extracellular signals into the gene and microRNA expression, and they follow hierarchically controlled gene expression which are spatiotemporally expressed during lineage progression and commitment. The determination of the myotome and dermomyotome are under the regulation of *Tbx6, ripply1*, and *mesp-ba* (Windner and Dorris, 2010). Wnt family proteins play a key role in the development of dermomyotome and myotome through canonical activation of beta-catenin/TCF transcriptional complex or through different non-canonical pathways. Determination and terminal differentiation of muscle cells are governed by a network of four MRFs: myogenic factor 5 (*MYF5*), muscle-specific regulatory factor 4 (*MRF4*; also known as *MYF6*), *MyoD* and *Myogenin* (Braun and Gautel, 2011). Another set of key genes involved in regulation of proliferation and commitment of myogenic precursor cells is paired-homeobox transcription factors *Pax3* and *Pax7* (Bentzinger et al., 2012). *Wnt* proteins are important for the expression of *Pax3, Pax7, MyoD, MRF4, Myf5* and *Myogenin* which are the most important group of transcription factors involved in regulation of muscle development (Tong et al., 2009). *Myf5* is the first MRF expressed during embryonic development, in the paraxial mesoderm and at a later stage along with other MRFs aids in the formation of the myotome (Braun and Gautel, 2011). *MYOD* and *MYF5* are muscles specific transcription factors which are cross-regulatory and in a redundant fashion, act upstream of *Myogenin* and *MRF4*. (Francis-West et al., 2003). Disruption

of the expression of *MYOD* and *MYF5* negatively impacts skeletal muscle formation depending on the severity of the disruption (Francis-West et al., 2003). *Myogenin* and *MRF4* are more directly involved in the differentiation process and trigger the expression of myotube-specific genes. *Myogenin* is essential for the terminal differentiation of committed myoblasts and is the signal for cells to exit the cell cycle in case of satellite cells (Bentzinger et al., 2012). *MRF4* seems to have a dual role of aiding differentiation and innervation, and acting as differentiation gene in undifferentiated proliferating cells. Studies using mice models have demonstrated *Pax3* plays a key role in activating *Myf5* by controlling the expression of *Dmrt2*. Mice models that evaluated impacts of depleted *Pax3* and *MYF5* showed loss of *MyoD* expression which indicates that *MyoD* expression depends on either *Pax3* or *Myf5* (Braun and Gautel, 2011). Sonic hedgehog is another factor that is important for the formation of myotome and expression of *Myf*5. Bone morphogenic protein (*BMPs*) signaling plays a key role in the regulation of myogenesis and maintaining the pool of progenitor cells. *BMPs* functions to expand the pool of myogenic progenitors before a further commitment is initiated and therefore delays the process of differentiation (Braun and Gautel, 2011)The balance between *Wnt, Shh,* and *BMPs* is essential for the regulation of *MyoD* and development of myotome. Notch signaling also plays a role in regulating myogenesis by preventing differentiation and promoting the expansion of progenitor cells through Delta 1 and Jagged ligands (Schuster-Gossler et al. 2007; Vasyutina et al. 2007). Notch signaling antagonizes the expression of *MyoD* in cooperation with the DNA binding protein *RBP-J* and *Hes1* (Jarriault et al. 1995). Another pathway that plays a key role in the regulation of myogenesis is the phosphatidylinositol 3-kinase/Akt pathway and the downstream genes such as *mTOR* and *FOXO1*. These pathways has a key role in regulating the metabolism, protein accretion, cell atrophy and cell cycle fate determination (Costello et al., 2013).

MRFs are also assisted by many other factors such as *PBX Homeobox 1* (*PBX*) and *Meis Homeobox 1* (*MEIS*) proteins. They function as heterodimers and act as cofactors with basic helix–loop–helix (bHLH) proteins such as MRFs and various homeobox transcription factors. Myocyte enhancer factor 2 (*MEF2*), which are members of this regulatory circuit, does not have myogenic activity but potentiate the function of MRFs through transcriptional cooperation. *MEF2* proteins interact directly with *MyoD* and is involved in the feed forward mechanism associated with MRFs (Francis-West et al., 2003). It also acts on *HDAC9* to act as a negative feedback loop and repress MEF2 activity (Radhakrishnan et al., 2015). Another group of genes called the sine oculis–related homeobox 1 (*Six1*) and sine oculis–related homeobox 4 (*Six4*) are considered to be the apex of the genetic regulatory cascade that directs dermomyotome progenitors toward the myogenic lineage (Bentzinger et al., 2012). *Six1* and *Six1* double mutants show a reduced expression of *Pax3*, *MyoD*, *Myogenin* and other myotomal markers (Braun and Gautel, 2011).

miRNAs are evolutionarily conserved, small noncoding RNAs that associate with the 3′ untranslated regions of target mRNAs to induce their translational repression or cleavage (Bartel, 2004). Recent studies suggest that miRNAs act as modulators of myogenic differentiation because some miRNAs such as *miR-1* and *miR-133* are absent from undifferentiated myoblasts and are strongly upregulated upon differentiation (Braun and Gautel, 2011). miR206 is one such mRNA that is induced by *MyoD* and can suppress the expression of *Pax3* and *Pax7* (Chen et al., 2010). Recently, analysis of mice deficient in *Myf5* and *MyoD* revealed a surprisingly specific requirement of *Myf5* for *miR-1* and *miR-206* expression (Braun and Gautel, 2011). Many other microRNAs are currently under investigation as potential candidates that could play a role in the process of myogenesis.

In higher order eutherian mammals, adult myogenesis involves hypertrophy or regeneration in the case of muscle injury. Adult myogenesis is different from embryonic, and fetal muscle development in that muscle regeneration in higher vertebrates requires an extracellular matrix for the formation of muscle fibers (Ciciliot and Schiaffino, 2010). Muscle tissue regeneration involves the recruitment of progenitor cells called satellite cells (Rudnicki et al., 2008). These cells have the capacity of asymmetric divisions for self-renewal and at the same time give rise to cells committed to myogenic lineage (Dumont et al., 2015). The molecular regulation of satellite cell differentiation is under the same factors that regulate differentiation of muscle fibers from myogenic precursor cells during prenatal development (Rudnicki et al., 2008). Briefly, muscle development is a complex process that occurs under the control of multiple regulatory factors, which are specific and critical for different stages of myogenesis.

Development of adipose tissue

Function and significance of adipose tissue

Adipose tissue develops at multiple locations inside the body in a time-dependent manner starting from the fetal period (Berry et al., 2013). The development of adipose tissue is dynamic in nature, and responsive to homeostatic and external cues and capable of expansion throughout the life of the individual (Du et al., 2010b; Berry et al., 2013). There are two types of adipose tissue that can be distinguished in higher order mammals: white adipose tissue and brown adipose tissue (Saely et al., 2012). The primary function of white adipose tissue is to store energy in the form of triacylglycerol when the intake of energy is more than the expenditure (Saely et al., 2012). Brown adipose tissue is involved in the dissipation of energy through the production of heat (Saely et al., 2012; Schulz and Tseng, 2013). In addition, adipose tissue has a significant

roles as an endocrine and paracrine tissue and is involved in mediating multiple physiological and pathological processes by secreting factors that control carbohydrate metabolism, inflammatory and immunological responses, reproductive function and angiogenesis (Schulz and Tseng, 2013; Berry et al., 2013). In recent years, adipose tissue has been identified to regulate appetite by secretion of factors such as adiponectin and leptin (Schulz and Tseng, 2013). Adiponectin has a crucial role in regulating glucose and fatty acid metabolism whereas leptin regulates hunger and helps in maintaining energy balance in the body (Schulz and Tseng, 2013). In addition, adipose tissue is important for thermal regulation in the body by acting as an insulator of heat. Furthermore, adipose tissue attaches the skin to the underlying tissue and protects body parts by acting as a cushion. The major depots of adipose in the body are subcutabeous, renal, intramuscular, pericardial and abdominal. Depending on the location of the adipose tissue, the function and impacts can vary. For instance, in livestock production where meat production is an integral part of the production process, intra-muscular adipose content plays a crucial role in the palatability, taste and shelf-life of meat (Du et al., 2010b).

Development and regulation of adipose tissue

Adipogenesis is the process of differentiation of mesenchymal stem cells into preadipocytes and subsequent secondary differentiation of preadipocytes into mature adipocytes filled with lipid. This process involves six stages: 1) mesenchymal precursors, 2) committed preadipocytes, 3) growth-inhibited preadipocytes, 4) mitotic clonal expansion, 5) terminal differentiation and 6) mature adipocytes (Lefterova and Lazar, 2009). Mature adipocytes then to increase in size by accumulating triglycerides. Adipogenesis is tightly regulated by different stimulatory factors such as proliferator-activated receptor γ (*PPAR γ*) and enhancer binding protein (*C/EBPs*) α, β and γ (Saely et al., 2012). C/EBPs induce the expression of PPAR γ which

dimerizes with retinoid X receptor (*RXR*) to promote adipose differentiation. *PPAR γ* and *C/EBPs* then induce each others expression in a positive feedback loop thereby maintaining the differentiated cell state (Rosen et al., 2002). Single transducers and activators of transcription (STATs), the transcriptional factor sterol-regulatory-element-binding-protein-1 (*SREBP1*) macrophage colony stimulating factor, fatty acids, prostaglandins, and glucocorticoids are other stimulators of adipogenesis (Saely et al., 2012; Schulz and Tseng, 2013). SREBPs are associated with the regulation of enzymes involved in the synthesis of cholesterol, fatty acid, triacylglycerol and phospholipids (Berry et al., 2013). Factors such as Wnt, various cell cycle proteins, several interferon regulatory factors IRFs (IRF3 and IRF4), B-cell factor 1 (*EBf1*) and GATA-binding protein-2 and -3 are also known to regulate the process of adipogenesis. Wnt signaling factors are inhibitors of adipogenic differentiation and inhibition of Wnt signaling is associated with increased adipose accumulation in rodents (Rosen and MacDougald, 2006). Inhibitors of adipogenesis include growth hormone, fibroblast growth factor, transforming growth factor β, notch ligands and pro-inflammatory cytokines (Rosen and MacDougald, 2006). These factors are in turn regulated by multiple interconnected internal and external signals that can have a significant impact on the expression of these tissues and thereby on adipogenesis.

Development of bone tissue

Bone significance and function

Bones are highly specialized tissues in the mammalian body that form the endoskeleton of vertebrates, protect the vital organs, and provide a structural framework to the body (Florencio-Silva et al., 2015). In addition, they provide a safe environment for the development and maintenance of marrow and bone marrow derived stem cells. They also act as reservoirs for

minerals, cytokines, and growth factors, which are essential for normal functioning of the body. Since bones have a crucial role in the operation of multiple systems in the mammalian body, their adequate development is critical for maintaining high productivity and general health of individuals (Florencio-Silva et al., 2015).

Bone development and regulation of bone development

Bone formation is a complex process that is regulated by interacting intrinsic and extrinsic factors. Extrinsic factors include prenatal and postnatal nutrition the animal receives and exposure to toxins (Lanham et al., 2008a; Goodfellow et al., 2010). The intrinsic factors are hormones and transcription factors that tightly regulate the process of bone formation from the fetal stage. Bone development is a continuous process, and the dynamic balance between formation and resorption determines the strength and functional capacity of the bones. Mesenchymal stem cells which are located in the inner layer of periosteum, endosteum and the vascular structures in the matrix play a vital role in maintaining the dynamic balance involved in necessary bone growth (Kirkham and Cartmell, 2012; Florencio-Silva et al., 2015).

Intramembranous ossification and enchondral ossification are the two process by which bones develop (Lanham et al., 2008b; Kirkham and Cartmell, 2012). Intramembranous ossification occurs by the stepwise conversion of mesenchymal tissue directly into bone. During the process, mesenchymal cells proliferate and condense into compact nodules which later change their morphology and become osteoblasts which are the committed precursor bone cells (Kirkham and Cartmell, 2012). Osteoblast cells then secrete the extracellular matrix and develop into osteocytes which are the mature cells within the bone that account for the majority of bone cells (Baat et al., 2005). Osteoblasts also secrete collagen and matrix that make up unmineralized

bone, called osteoid. Osteoclasts, another key cell in bone development and maintenance are multinucleated giant cells involved in resorption of bone (Teitelbaum, 2000). The differentiation of MSC into bone is tightly regulated by different intrinsic factors. Purinergic receptors are known to be involved in regulating stem cell differentiation and commitment into osteoblasts or adipocytes. Bone morphogenetic proteins (BMP2, BMP4, and BMP7) from the head of the epidermis are involved in the commitment of MSC to become bone cells (Hall, 1988). BMPs initiate the expression of Runt-related transcription factor 2 (*RUNX2*) gene in the MSC which is the master regulatory gene involved in transforming mesenchyme cells into osteoblasts (Kirkham and Cartmell, 2012). *RUNX2* is also involved in the involved in the expression of osterix, osteocalcin, osteopontin, and other bone-specific extracellular matrix proteins which are essential for the development of bone directly from MSC (Florencio-Silva et al., 2015).

Long bones, such as the femur, tibia, and humerus develop through the process of enchondral ossification during prenatal development and the process continues untill puberty (Mehta et al., 2002). During endochondral ossification, the mesenchymal cells differentiate into cartilage, and this cartilage is later replaced by bone (Horton, 1990). The first step in enchondral ossification involves the commitment of MSC into cartilaginous precursors and is under the control of two transcription factors *Pax1* and *Scleraxis (Kirkham and Cartmell, 2012).* The second stage involves the differentiation of committed MSC into chondrocytes. This process is governed by *N-cadherin, N-CAM* and *SOX9* which are crucial for the differentiation into chondrocytes and their maintenance (Lanham et al., 2008b; Kirkham and Cartmell, 2012). These chondrocytes then undergo proliferation followed by hypertrophy to become large chondrocytes. These hypertrophic chondrocytes are mineralized by calcium carbonate and are subsequently invaded by blood vessels. This is followed by the apoptosis of the chondrocyte, and as the

cartilage cells die, a group of cells that have surrounded the cartilage model differentiate into osteoblasts. The osteoblasts begin forming bone matrix on the partially degraded cartilage (Bruder and Caplan 1989; Hatori et al. 1995). Thus, cartilage is replaced by bone to complete the bone formation process. Mineralization of bone occurs in the later stage of bone development and continues throughout life as bone remodelling by osteoblasts and osteoclasts is a continuous process. Mineralization of bone is influenced by various extrinsic factors, such as nutrition and intrinsic factors, such as hormones (Florencio-Silva et al., 2015). Bone formation and resorption are continuous processes that starts very early during gestation, continue throughout life, are tightly regulated, and influenced by multiple genetic, physiological and environmental factors.

General effects of poor maternal nutrition on offspring

In many species, such as sheep, the phase of in-utero growth is critical because the animal spends a considerable amount of their life, untill maturity, in the prenatal stage (Gerrard and Grant, 2006). Therefore, optimal growth during this period is critical. Many factors can influence the growth and development of organisms during this critical period of growth. As discussed earlier, maternal nutrition is one such factor that has significant impacts on the growth during the in-utero period. Therefore, alterations in maternal nutrition are broadly covered by the term poor maternal nutrition and can impact the fetus growth and development. The impacts of poor maternal nutrition are dependent on the stage of gestation and the severity of the alterations in the maternal nutrition (Wu et al., 2006).

Poor maternal nutrition increases neonatal morbidity and mortality in addition to altering postnatal growth (Reynolds et al., 2010). In addition, poor maternal nutrition affects body composition as a whole and alters the composition of specific tissues (Wu et al., 2006; Ford et

al., 2009). Evidence from multiple studies shows that exposure to poor maternal nutrition detrimentally affects the prenatal and postnatal growth trajectory of eutherian mammals (Kind et al., 2005; Wu et al., 2006). Poor maternal nutrition during gestation is known to negatively affect the development of various tissues in the body by altering endocrine growth factors, such as Insulin like growth factor I (IGF-I) (Ford et al., 2009; Hoffman et al., 2014).

Poor maternal nutrition disrupts the state of homeostasis in offspring causing metabolic disorders, and is known to negatively affect growth and function of nearly all organ systems (Caton and Hess, 2010; Reynolds et al., 2010). For instance, in rodents, exposure to poor maternal nutrition programs the mitochondrial function and downregulates the function of glycolytic enzymes in offspring hepatic tissues making these animals more prone to hyperlipidaemia and type 2 diabetes in adult life (Desai et al., 1997; Guillou et al., 2014). The detrimental effects of poor maternal nutrition can be attributed to altered partitioning of nutrients during the critical process of growth and development that occurs during gestation. There is evidence that the effects of poor maternal nutrition during gestation can persist in to adult life of offspring and transmitted into next generation through various mechanisms such as epigenetic modifications (Langley-Evans, 2006; Simmons, 2011). In general, available evidence suggests that poor maternal nutrition has permanent negative effects on growth and development of fetus and neonates, pre-weaning survival, postnatal efficiency, feed utilization, meat quality and lifetime health of animals (Wu et al., 2006; Caton and Hess, 2010; Du et al., 2013). The impacts of poor maternal diet on specific organs and tissues are discussed in detail below.

Effects of maternal nutrition on organ development

Organogenesis is a critical process that occurs in-utero. Lack of adequate supply of nutrients to the fetus or altered partitioning of nutrients during this window of development and growth might detrimentally impact growth, development, and functions of organs in later life (Reynolds et al., 2010). The prenatal development of organs is, therefore, critical since a major portion of organ development occurs before birth. In sheep, maternal nutrient restriction (lower metabolizable energy) from day 30 to day 80 of gestation affects the development of liver by increasing liver weights and increasing adipose content in the liver (Hyatt et al., 2008). These changes in liver subsequently altered key metabolic pathways involved in carbohydrate and lipid metabolism leading to disorders including hyperglycaemia and hyperlipidaemia (Hyatt et al., 2008). In rats, under- and over-nutrition leads to alterations in pancreatic beta cell development and function, leading to a reduction in insulin production ultimately affecting glucose metabolism (Reusens et al., 2011). In higher order mammals, the heart is a relatively mature organ at birth with limited ability for regeneration of the heart at birth. Therefore, environmental factors, such as poor maternal nutrition, that alter the timing of cardiomyocyte binucleation in late gestation are likely to have long-lasting consequences for heart growth and function (Krishnan et al., 2014). Poor maternal nutrition leads to altered development and growth of heart and increasing lipid accumulation in heart and oxidative stress in rats (Tarry-Adkins et al., 2013), making offspring prone to cardiovascular disorders later in life. Also, exposure to intra-uterine growth retardation (IUGR) resulted in an increase in relative heart weight, a decrease in DNA content in the heart at birth, and increased the vulnerability to ischemia reperfusion injury. Exposure to poor maternal nutrition is known to affect the normal development and physiology of nephrons such as reduction in nephron numbers leading to impaired kidney functions in later life in several animal species (Wood-Bradley et al., 2015; Luyckx and Brenner, 2015). Recent

studies using ovine models have demonstrated that altered intrauterine environment, especially during the key phases of nephrogenesis can impact kidney function and lead to adult renal diseases such as albuminuria and glomerulosclerosis (Jackson et al., 2012; Meyer-Gesch et al., 2013). Although it is not clear how adrenal gland function and development are altered as a result of exposure to poor maternal nutrition, studies using pig models show that the poor maternal diet influences maternal-fetal cortisol regulation (Bloomfield et al., 2003; Kanitz et al., 2012).

Effects of maternal nutrition on muscle development and function

The fetal period is critical because the number of muscle fibers is determined by birth or early postnatal period in most eutherian mammals. Prenatal muscle growth and development in eutherian mammals occurs in a biphasic nature prenatally, namely primary myogenesis and secondary myogenesis (Francis-West et al., 2003; Du et al., 2011; Bentzinger et al., 2012). Primary myogenesis is the development of primary myofibers from myogenic precursor cells and secondary myogenesis involves the development of smaller secondary myofibers using primary myofibers as template (Bentzinger et al., 2012). Moreover, studies have reported that poor maternal nutrition can alter the hypertrophy of muscle fibers, fiber type composition, collagen content and intramuscular adiposity during the postnatal life of offspring (Kind et al., 2005; Zhu et al., 2008; Tong et al., 2009; Reed et al., 2014; Xu et al., 2014). Development of skeletal muscle is especially vulnerable to nutritional deficiency compared to other tissues, owing to muscle mass being lost at the expense of brain and heart development in utero. Although this adaptation might be advantageous as a short-term adjustment, it is now understood that the

negative impacts on muscle development at early stages can predispose the animals to metabolic diseases in its later life (Yan et al., 2011; Du et al., 2013). Therefore, it is important to understand how maternal nutrition during these stages of muscle development negatively impacts growth and development of this tissue.

The type and extent of alterations in the muscle depend on the stage of exposure to poor maternal nutrition, severity of restriction or over-feeding and type of poor maternal diet (Bee, 2004; Zhu et al., 2004; Huang et al., 2010; Woo et al., 2011; Yan et al., 2013b). For instance, cross-sectional area of semitendinosus muscle from offspring exposed to under- and over-feeding is altered in comparison with controls, depending on the stage of postnatal growth (Zhu et al., 2008; Reed et al., 2014). In other studies that evaluated the impacts of poor maternal nutrition on muscle development, although the effects of poor maternal nutrition on muscle weight are consistently negative, effects of poor maternal nutrition during late gestation on offspring muscle morphometrics such as muscle fiber type, fiber number, and protein accretion vary depending on the age of offspring (Gondret et al., 2005; Tygesen et al., 2007; Sen et al., 2016). It is also known that the effect of poor maternal nutrition during gestation on growth and development are dependent on the type of nutrition and stage of gestation (Zhu et al., 2008; Reed et al., 2014). Poor maternal nutrition, either under or over-feeding during gestation reduces protein accretion and thereby muscle mass in the postnatal life of offspring (Daniel et al., 2007; Du et al., 2015). In pigs, maternal low energy diet is known to decrease the muscle growth, myofibers number as well as alter muscle fiber type and morphometry (Bee, 2004). Studies using ovine models of maternal under-nutrition based on restricted protein reported fiber specific alterations to the muscles, in addition to reducing the area and altering the diameter of neuromuscular junctions (Confortim et al., 2015). In addition to reducing number of fibers

developed poor maternal nutrition also reduces the ratio of secondary to primary fibers (Zhu et al., 2004; Quigley et al., 2005). In pigs, maternal overfeeding also lead to changes in post-natal fiber type composition of LM and STN muscles (Bee, 2004). Since muscle fiber development occurs prenatally, exposure to poor maternal nutrition limits the postnatal compensatory growth of muscles (Wu et al., 2006). In addition, poor maternal nutrition reduces DNA concentration in muscles by affecting satellite cells thus contributing to reduced postnatal muscle growth (Greenwood et al., 2002). Studies conducted using ovine models of poor maternal nutrition have demonstrated that lipid content is increased in the offspring muscle and expression of key genes involved in the development of muscle such as *MyoD, Myogenin* and *MRF4* are reduced as a result of exposure to poor maternal nutrition (Zhu et al., 2008; Tong et al., 2009; Reed et al., 2014; Hoffman et al., 2016b). Decrease in myogenic factor MyoD, and increased expression of *TGFβ1* as result of changes in Wnt/β-catenin signalling pathway, decreased AMPK activity evoked by *TNF-α*, increased activity of NF-kappaB in response to inflammation have been reported in multiple species as a result of exposure to maternal obesity/overfeeding (Zhu et al., 2008; Tong et al., 2009; Grabiec et al., 2012). In addition, the negative impacts on the development can impact the metabolism and functioning of other organs such as liver and kidney (Argiles et al., 2001).

Fetal skeletal muscle development involves myogenesis, adipogenesis, and fibrogenesis, which are all derived from mesenchymal stem cells (Du et al., 2010a). Maintaining a balance between these tissues is critical for proper function and optimum meat production. Intramuscular fat (marbling) is crucial for meat palatability and attaining desired texture (Du et al., 2011; Du et al., 2015). But excess of intramuscular fat can affect the nutritional quality and keeping quality of meat (Hathwar et al., 2011). Fetal and neonatal stages are major stages for generation of

intramuscular adipocytes which provide the sites for intramuscular fat accumulation or marbling formation during fattening (Tong et al., 2008). Increased fibrogenesis of muscle is also not a desired trait in meat production as it affects the texture and palatability of meat (Du et al., 2011). Increased intra-muscular adiposity and fibrous tissue development have been observed as a result of exposure to maternal under- and over-feeding during gestation in sheep (Tong et al., 2008; Reed et al., 2014). This might be due to shifting and commitment of MSCs from myogenesis to adipogenesis and fibrogenesis (Du et al., 2011). This alteration will result in increased intramuscular fat and connective tissue, as well as reduced numbers of muscle fiber and/or diameter, all of which have lasting negative effects on offspring muscle function and properties (Du et al., 2011). Increased adiposity, fibrous tissue content and decreased muscle development can be through multiple mechanisms which includes reduction in *Wnt* signalling, enhanced *TGFβ*, inhibition of AMP-activated protein kinase, which promotes adipogenesis and epigenetic modification through polycomb group proteins (Tong et al., 2008; Zhu et al., 2008; Huang et al., 2010; Du et al., 2011). In brief, poor maternal nutrition negatively impacts the development of muscles by affecting hyperplasia and hypertrophy in addition to increasing the adiposity in these tissues. A potential mechanism behind this might be the alterations to the expression of various genes involved.

Several critical signaling pathways are involved in the differentiation and maturation of MSC into myocytes and adult myofibers (Francis-West et al., 2003; Bentzinger et al., 2012). Prenatal myogenesis occurs throughout the major portion of gestation and nutritional insults occurring at periconception period is also known to impact myogenesis and factors involved in regulating myogenesis (Quigley et al., 2005; Sen et al., 2016). The differentiation of MSC into myocytes and then adult muscle fibers occurs under the control of myogenic factors such as

Pax7, *Myf5*, *MyoD*, *Myogenin*, and *Myostatin* (Bentzinger et al., 2012). These factors can be affected by the plane of maternal nutrition (Yan et al., 2013b; Reed et al., 2014; Penagaricano et al., 2014; Raja et al., 2016; Hoffman et al., 2016b). Factors such as *FOXO, PGC, SOCS, TGF- β* are known to have key roles in development and functioning of muscle. Enhanced, *TGF-β* signalling, and increase in *Smad7* and *fibronectin* is reported to contribute to increased fibrogenesis in ovine fetal skeletal muscle in offspring of obese dams at late gestation (Huang et al., 2010). Increased fibrogenesis at early stage of fetal development is expected to negatively affect the properties of offspring muscle because muscle fibrosis is an indicator of aging and muscle damage (Tong et al., 2008). Insulin signaling and *Wnt* signaling pathways are also known to be key contributors to development, protein accretion, and proteolysis in muscles. Maternal obesity is known to downregulate β-catenin signalling and affect muscle development in fetal sheep (Tong et al., 2009). Studies done using sheep models have shown there is a decrease in fetal glucose, insulin and IGF-I levels in underfed ewes at day 135 of gestation and this may have compromise fetal muscle growth and metabolism (Osgerby et al., 2002). Studies that have evaluated fetal muscle development have reported downregulation of insulin signalling in muscles as a result of exposure to maternal over-feeding (Zhu et al., 2008). In addition, maternal under-feeding in sheep also reduced the insulin signalling in male offspring at 210 days of age postnatally (Costello et al., 2013). Studies have evaluated the role of skeletal muscle transcripts in the development of muscles but little has been done to understand the impacts of poor maternal nutrition on these critical muscle transcripts. Therefore, determining global differential gene expression of skeletal muscle of offspring exposed to poor maternal nutrition during gestation will provide insights into mechanisms involved in the negative effects of poor maternal nutrition on muscle development.

Effects of maternal nutrition on bone development and function

Although genetic factors have the most important role in regulating bone development, environmental factors such as nutrition can alter the peak bone mass by 20 to 30 percent (Eastell and Lambert, 2002). Maternal undernutrition is known to decrease the weight of femur and reduce the process of mineralization of tibia in rats (Mehta et al., 2002; Tygesen et al., 2007). In pigs, maternal vitamin D deficiency is known to decrease bone mineral content (BMC) during intrauterine life, which increases the risk of hip fractures later in life (Schlussel et al., 2010). Maternal protein insufficiency during pregnancy in rats led to offspring that had femoral heads with thinner and less dense trabeculae which in turn were structurally weaker when mechanically tested compared with that of control groups (Lanham et al., 2008 a,b). In rats, reduction in BMC and bone area are observed in offspring from dams fed a low protein diet during gestation in comparison with offspring fed a control diet (Oreffo et al., 2003; Lanham et al., 2011). Maternal high-calorie intake or obesity arising from over-nutrition can also impair skeleton development of offspring. Reduced bone volume to total volume ratio (BV:TV) in the femur as well as increased bone marrow adiposity is observed in adults born to high-fat diet fed mothers compared with adults born from mouse dams fed standard chow, indicating fetal programming in the uterus during gestation (Lanham et al., 2010). Since bones arise from MSC and they are known to be impacted by maternal diet, one possible mechanism behind the alterations in bone parameters might be due to the alterations in the function, proliferation and/or differentiation of MSC (Oreffo et al., 2003; Devlin and Bouxsein, 2012)

Effects of maternal nutrition on adipose development and function

Several studies have demonstrated that poor maternal nutrition during gestation increases adiposity in the offspring. Multiple epidemiological and animal studies have concluded that maternal under- and over-nutrition lead to increased adiposity in the early postnatal and adult life of offspring (Fiorotto et al., 1995; Kind et al., 2005; Long et al., 2010; Ware et al., 2015). Studies done using ovine models found that subcutaneous fat is increased by 100 % in offspring from obesogenic diet fed mothers in comparison with offspring from control diet fed mothers (Ford et al., 2009). Male lambs born to over-nourished lambs had increased perirenal and omental adipose depots weights in comparison with the controls lambs (Long et al., 2015) Interestingly, maternal under-nutrition has a similar effect in sheep with increased adiposity in offspring when compared with control postnatally (Ford et al., 2007). However, it is to be noted that fetal adipose tissue response to suboptimal maternal food intake at defined stages of development differs between location of the adipose depots (Symonds et al., 2016). Nutritional epidemiological studies that focused on impacts of poor maternal nutrition on development of adiposity have also reported a positive correlation between the poor maternal nutrition and obesity in early childhood and adult life of offspring (McMillen and Robinson, 2005; Godfrey et al., 2010; Varadinova et al., 2015).

Alterations to expression of key genes that regulate adipogenesis might be one mechanism behind the observed changes in adiposity in offspring from mothers exposed to poor maternal nutrition. At 89 days of gestation, fetus from under-nourished ewes had a reduction in the expression of *IGF-1, IGF-2* and *IGF-2R* mRNA compared with controls (Wallace et al., 2015). Uteroplacental insufficiency increased the expression of *PPARγ* in adolescent rats and *PPAR gamma* target genes in comparison with controls and these changes co-related with increased visceral adiposity in these animals (Joss-Moore et al., 2010). RNA-seq analysis conducted on fetal sheep adipose tissue also revealed that maternal diet composition can affect the

key pathways and differential expression of genes involved in regulation of adipose tissue development and function (Penagaricano et al., 2014). Recent studies also indicate that microRNA is intensively involved adipogenic differentiation from mesenchymal stem cells, and epigenetic changes such as DNA methylation are expected to alter development of adipose tissue during fetal growth (Yan et al., 2013b)

Increased adiposity and obesity are known to make individuals more prone to diseases, such as metabolic syndrome, type 2 diabetes and cardiovascular disease in later life (Rkhzay-Jaf et al., 2012). This, in turn, can impact the life expectancy as well as compromise the quality of life, in affected individuals. In addition, increased fat production can have an impact on the production of meat as well as overall health of animals (McMillen and Robinson, 2005; Du et al., 2010b). Although intra-muscular adipose tissue may increase the palatability and flavor of meat, consumption of high-fat meat might not be ideal to the consumer because of health concerns (Hathwar et al., 2011). Moreover, studies have suggested that the development of adipose may be at the cost of reduced bone and muscle development which might indirectly contribute to disorders in later life of the individual (Devlin and Bouxsein, 2012). The increased development of adipose at the cost of muscle might reduce the overall quality of the meat produced as well as the shelf life of meat and meat products (Du et al., 2011; Du et al., 2015).

Potential mechanisms behind the impacts of maternal diet on offspring bone, fat, and muscle

Role of MSC in bone and adipose development and maintenance

As mentioned previously, bones originate from MSC which are multipotent stromal cells. In addition to bone, MSC can develop into muscle, adipose, cartilage, tendons and ligaments

(Ohishi and Schipani, 2010). Among different sources of MSC, such as placenta, adipose tissue, lungs, blood, dental pulp, periodontal ligament of teeth, umbilical cord and bone marrow, the most common source of MSC is bone marrow, where they are immersed in the stroma (Caplan, 2015). MSC are characterized by adherent spindle-shaped cells capable of forming colonies that can self-renew and maintain their properties (Friedenstein et al., 1970). The International Society of Cellular Therapy (2013) has listed the minimum criteria for defining MSC, which state that they can adhere to a plastic surface in standard culture medium, and should have multipotent properties to differentiate into osteoblasts, adipocytes, and chondrocytes, in vitro. This is important, since these are the criterion used for the identification of isolated MSC in vitro.

The differentiation, proliferation, migration and maturation of MSC are tightly regulated by multiple genetic and environmental factors (Caplan and Hariri, 2015). Alterations in these functions can affect the development of tissues that arise from the MSC. The alterations in the tissue are observed because adult stem cells are remarkably malleable and exhibit a high degree of plasticity. Changes in genetic and environmental factors can affect the differentiation of MSC into one lineage versus another (Devlin and Bouxsein, 2012). Differentiation of MSC is a two-step process, which involves lineage commitment and maturation (Bianco et al., 2001; Rosen and MacDougald, 2006; Ohishi and Schipani, 2010). Recent studies have shown that critical signaling pathways involved in the lineage commitment of MSC can be influenced by environmental factors during the process of development (Sawada et al., 2006; Du et al., 2010b; Caplan, 2015; Caplan and Hariri, 2015).

MSC reside in specific locations in the body, but can be signaled to differentiate into new cells types during times of cell injury and to proliferate to maintain MSC numbers (Sawada et al., 2006; Ohishi and Schipani, 2010; Caplan, 2015). However, the function of MSC can be

influenced by several factors, including the quality of maternal diet in utero. Function and differentiation of progenitor stem cells are altered by poor maternal nutrition, and this might be a mechanism by which body composition is altered (Oreffo et al., 2003; Woo et al., 2011; Devlin and Bouxsein, 2012; Yan et al., 2013b). Poor material nutrition is known to impact the MSC cells by favoring their differentiation into one lineage versus another. Specifically, the differentiation of MSC to adipose versus bone is proposed as a reason behind reduced bone and increased adipose in animals exposed to poor maternal nutrition. Oreffo et al. (2009) using a rodent model demonstrated that the proliferative ability of MSC is reduced in offspring from the overfed mothers in comparison with control diet fed mothers. In addition to the differentiation into different tissues, MSC also has a role in the postnatal maintenance of tissues such as bone, which develop from them (Rosen and MacDougald, 2006; Ohishi and Schipani, 2010; Devlin and Bouxsein, 2012; Caplan, 2016). The reduced proliferation of these tissues in their adult life might adversely impact the maintenance of these tissues and could be a contributing factor to disease such as osteoporosis.

Role of satellite cells

Skeletal muscle satellite cells are adult muscle-derived stem cells that are critical in life-long maintenance and regenerative potential of muscles. Multiple studies have demonstrated the presence of these cells between the basement membrane and sarcolemma of skeletal muscle (Rudnicki et al., 2008). It is clear that poor maternal nutrition negatively impacts muscle development and postnatal muscle growth. Poor maternal nutrition is known to alter function and gene expression in satellite cells. Maternal nutrient restriction during gestation in sheep is known

to alter the temporal expression of key muscle regulatory factors in these cells such as *MyoD* and *Myogenin* (Raja et al., 2016). Also, studies in mouse models demonstrated that diet-induced obesity lead to attenuated muscle regeneration, as indicated by prolonged necrosis, delayed expression of *MyoD* and *Myogenin*, increased collagen content, and persistent embryonic myosin heavy chain expression (Dsouza et al., 2014). In addition, factors such as *IGF-1, TGFβ, and Fibroblast growth factor (FGF1)* which are known to have an impact on satellite cell functions are all altered as a result of exposure to poor maternal nutrition. Since these factors are important for the proliferation, differentiation and function of satellite cells, alterations to these factors might have an impact on satellite cells and subsequently on the development and regeneration of muscles. Therefore, alterations to satellite cell function might be a contributing factor behind the altered development of muscles and muscle regeneration observed in offspring exposed to poor maternal diet during gestation.

Alterations to protein and gene expression

There is growing evidence that nutritional perturbations during different stages of pregnancy can modify gene expression in various tissues, which, in turn, could have important implications for the development and function of these tissues. Adapting and controlling the expression of genes and proteins might be a mechanism to enable the fetus to adapt to its environment at birth (Wu et al., 2006; Langley-Evans, 2006). There are multiple reports across different species that exposure to poor maternal nutrition can alter the gene and protein expression of factors involved in organogenesis, adipogenesis, myogenesis, bone development and stem cell function (Han et al., 2004; Wu et al., 2006; Hyatt et al., 2008; Simmons, 2011; Hoffman et al., 2016b). For instance, in rodents, it has been reported that angiotensin type II (AT2) receptor is modified by feeding a low-protein diet in utero (McMullen et al., 2004).

Moreover, studies in ovine models that evaluated the impacts of maternal diet on renal development found altered expression of genes associated with renal endoplasmic reticulum (Lloyd et al., 2012). In addition, maternal diet is known to alter the expression of genes involved in muscle development, functioning and metabolism in ovine models of poor maternal nutrition (Tong et al., 2008; Zhu et al., 2008; Reed et al., 2014; Hoffman et al., 2016b). In sheep, over-nutrition during pregnancy induced over expression of genes regulating adipogenesis and lipogenesis in fetal perirenal fat tissue, which subsequently may result in increased adiposity in later life (Muhlhausler., 2004). Furthermore, it has also been reported that the gene expression of circulating growth factors in sheep has been altered because of exposure to maternal over-nutrition during gestation (Hoffman et al., 2014). These alterations might be indicative of the fetal programming in offspring exposed to poor maternal nutrition and may be a potential mechanism of the changes in tissue development observed in individuals exposed to poor maternal nutrition at birth.

Epigenetics

Epigenetics refers to the changes to an individual's genetic code that can alter gene expression without changing the DNA sequence, and are passed from one cell generation to the next (Feeney et al., 2014). Epigenetics is found to have a key role in altered fetal programming occurring as a result of exposure to poor maternal nutrition (Feeney et al., 2014; Lee, 2015; Sincennes et al., 2016). Two key mechanisms involved in the epigenetic modifications are histone modification and changes in DNA methylation. For DNA methylation, when a methyl group is added to the cytosine base that is located beside a guanine base (CpG site), the expression of the gene is reduced, because of silencing of gene and protein expression is reduced. Studies in rats have shown that deficiency of certain nutrients such as choline and folic

acid reduces the methylation of *DNA methyltransferase 1* and leads to the over expression of these enzymes(Imbard et al., 2013; Bhargava and Tyagi, 2014). This, in turn, leads to the increased global methylation in the offspring from mothers exposed to the choline or folic acid deficiency when compared with controls (Imbard et al., 2013; O'Neill et al., 2014; Rochtus et al., 2015). This programming can be caused by various factors and can occur as early as 5 days of gestation in rats (Maloney et al., 2011). Studies using rodent models have demonstrated that maternal dietary fat significantly increased DNA methylation status of the leptin gene, which is associated with the control of energy balance and satiety (Zheng et al., 2015). Maternal underweight and overweight during pregnancy altered methylation patterns in the children who develop later infant adiposity (Agha et al., 2016).

Several studies have demonstrated that the impacts of poor maternal nutrition are transmitted across generations (Simmons, 2011; Feeney et al., 2014; Wood-Bradley et al., 2015). Epigenetic modifications such as alterations to methylation patterns and histone modifications have been associated with this transgenerational transmission of induced phenotype as a result of exposure to poor maternal nutrition. For instance, daughters and granddaughters of women exposed to maternal nutrient restriction during Dutch hunger winter, showed decreased birth weight (Stein and Lumey, 2000; Painter et al., 2005). Similarly, the chance of occurrence of cancer is greater in later life of the children and grandchildren of women exposed to the synthetic estrogen and endocrine disruptor diethylstilbestrol (DES) (Newbold et al., 2006). Using the protein-restricted model of maternal undernutrition, studies have demonstrated that maternal protein restriction of F0 generation resulted in elevated blood pressure, endothelial dysfunction and insulin resistance in F1 and F2 generation, even after the F1 generation was given a normal control diet (Newbold et al., 2006). Another remarkable study that investigated the methylation

pattern of F1 and F2 offspring of F0 rats fed a protein-restricted diet during gestation found that the changes to methylation in both F1 and F2 restricted offspring were similar (Burdge et al., 2006). The negative impacts were observed even though F1 generation received adequate protein during gestation. These changes observed across generations suggest that the impacts of poor maternal nutrition are transgenerational and permanent and epigenetic modifications might be the potential mechanism for the permanent transgenerational impact associated with poor maternal nutrition.

Rationale

Studies on multiple species have shown that growth, development, metabolism, and multiple organ systems of offspring are affected are affected by poor maternal nutrition. This altered growth, development, and sub-optimal functioning of the offspring as a complete organism, organ systems, or specific tissues can lead to a reduction in the general health and production in animals. Most of this evidence comes from studies in animals during their postnatal life, some as early as birth. Therefore, there is a need to evaluate the effects of poor maternal during the growth and development in-utero and early postnatal time points. This will help to gain a better understanding of perinatal growth and development during gestation and potential mechanisms involved causing these changes, especially fetal programming. Although few studies that evaluated the impacts of maternal nutrition on prenatal development found under- or over- feeding during gestation can negatively impact offspring, there were considerable variations in experimental design. Moreover, most of the studies investigated the impacts of either over- or under-feeding, but not both. In most studies, exposure to poor maternal nutrition was during late gestation and this necessitates evaluating effects of maternal under- and over-feeding from multiple time-points in-utero with exposure to poor maternal nutrition starting early

in the prenatal life. Therefore, in light of recent findings about stage dependent effects of poor maternal nutrition affecting carcass quality in lambs and developmental origins of many adult diseases, there is a need to evaluate the effects of poor maternal nutrition during various stages of development in-utero and early post-natal time points.

Hypotheses

We hypothesized that under- or over-feeding ewes during gestation will affect the growth and development of offspring through phenotypic and modifications in gene expression. This general hypothesis was further divided into four separate hypotheses described below.

1. Under- or over-feeding ewes during gestation will decrease bone development, increase fat development and alter proliferation, differentiation and metabolism of MSC in offspring.

2. Under- or over-feeding ewes during gestation will decrease body weight and weight of organs of fetuses and lambs.

3. Under- or over-feeding ewes during gestation will reduce growth and development of muscles and alter global gene expression in the muscle of offspring.

4. During fetal development, muscle fiber CSA of LM, STN and TB will increase over time and the global gene expression of key genes involved in regulating development and metabolism of muscles will be altered.

CHAPTER 1: EFFECTS OF POOR MATERNAL NUTRITION DURING GESTATION ON BONE DEVELOPMENT AND MESENCHYMAL STEM CELL ACTIVITY IN OFFSPRING

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Abstract

Poor maternal nutrition impairs overall growth and development of offspring. These changes can significantly impact the general health and production efficiency of offspring. Specifically, poor maternal nutrition is known to reduce growth of bone and muscle, and increase adipose tissue. Mesenchymal stem cells (MSC) are multipotent stem cells which contribute to development of these tissues and are responsive to changes in the maternal environment. The main objective was to evaluate the effects of poor maternal nutirtion during gestation on bone and MSC function in offspring. Thirty-six ewes were fed 100%, 60%, or 140% of energy requirements (NRC, 1985) beginning at day 31 ± 1.3 of gestation. Lambs from ewes fed 100% (CON), 60% (RES) and 140% (OVER) were euthanized within 24 hours of birth (1 day; n = 18) or at 3 months of age (n = 15) and bone and MSC samples were collected. Dual X-ray absorptiometry was performed on bones obtained from day 1 and 3 months. Proliferation, differentiation, and metabolic activity were determined in the MSC isolated from lambs at day 1. Data were analyzed using mixed procedure in SAS. Maternal diet negatively affected offspring MSC by reducing proliferation 50 % and reducing mitochondrial metabolic activity. Maternal diet did not alter MSC glycolytic activity or differentiation in culture. Maternal diet tended to decrease expression of P2Y purinoreceptor 1, but did not alter expression of other genes involved in MSC proliferation and differentiation. Maternal diet did not alter bone parameters in offspring. In conclusion, poor maternal diet may alter offspring growth through reduced MSC proliferation and metabolism.

Further studies evaluating the potential molecular changes associated with altered proliferation and metabolism in MSC due to poor maternal nutrition are warranted.

Introduction

In multiple species, poor maternal nutrition during gestation impairs fetal and postnatal growth and development. However, the specific mechanisms are not clear (Lanham et al., 2008a; Reynolds et al., 2010). Poor maternal nutrition can result from excess or reduced nutrient intake including energy, protein, and micronutrients in the diet. Poor maternal nutrition during gestation is known to reduce fetal growth (McMillen and Robinson, 2005; Wu et al., 2006; Reynolds et al., 2010), impair muscle development (Du et al., 2011; Reed et al., 2014), reduce bone density (Lanham et al., 2008a; Lanham et al., 2008b), increase fat accretion (Fiorotto et al., 1995; Du et al., 2010b; Du et al., 2011), alter metabolism (Wu et al., 2006; Reynolds et al., 2010; Guillou et al., 2014), and impair stem cell function (Oreffo et al., 2003; Raja et al., 2016) in the offspring.

Previous work by our laboratory group and others demonstrates that poor maternal nutrition alters muscle, bone, and fat development in offspring during pre- and post-natal growth. Specifically, using a sheep model, restricted- and over-feeding during gestation altered muscle CSA in offspring at birth and three months of age, and increased adipose content of muscle (Reed et al., 2014). Additionally, subcutaneous fat was reduced in offspring from restricted-fed ewes (Hoffman et al., 2016a). The known effects of maternal diet on fetal and postnatal bone of the offspring are limited; however, maternal diet may program offspring for reduced bone later in life (Devlin and Bouxsein, 2012). Poor maternal nutrition during gestation not only has a direct effect on offspring nutrient availability, but also creates an environment of stress that can lead to long-term or permanent effects on tissue development and stem cell function (5,14). Thus, the negative effects on offspring are well documented, but mediators of these effects are

not well established.

Mesenchymal stem cells are multipotent stem cells that contribute to the development of several tissues of mesenchyme origin, including muscle, bone, and adipose, as well as maintenance and repair of these tissues through adulthood (Caplan, 2015). Additionally, MSC are key components of the bone marrow niche, which are responsive to hormonal and metabolic changes in the whole body (Reagan and Rosen, 2016). Factors, such as maternal diet, may initiate MSC to differentiate into one lineage versus another (e.g., adipose vs. bone) (Devlin and Bouxsein, 2012). This diversion could be a potential mechanism by which poor maternal diet alters the development of muscle and adipose tissue as well as bone. Furthermore, this may contribute to the increased risk of obesity and osteoporosis later in life in the offspring (Devlin and Bouxsein, 2012). For example, in a rodent model, MSC of offspring from mothers fed a low protein diet during pregnancy exhibited reduced proliferation and ability to differentiate into bone forming cells (osteoblasts) at 8 weeks of age (Oreffo et al., 2003). Maternal programming of MSC may contribute to altered muscle, bone, and adipose tissue growth in offspring. However, the specific effects of poor maternal nutrition due to restricted- or over-feeding on MSC function and metabolism need to be evaluated. The objective of the current study was to determine the effects of poor maternal nutrition on bone development and MSC proliferation, differentiation and energy metabolism in offspring at birth and at three months of age. We hypothesized that poor maternal nutrition during gestation would reduce bone density and impair MSC function and metabolism in the offspring.

Materials and methods

Animals

All animal protocols were reviewed and approved by the University of Connecticut

Institutional Animal Care and Use Committee. Thirty-six multiparous ewes from the University of Connecticut sheep flock (25 Dorsets, 7 Shropshires, and 4 Southdowns) were estrus synchronized (Knights et al., 2001) with progesterone controlled intravaginal drug release devices (Pfizer Animal Health; New York, NY, USA) and Lutaylase (Pfizer Animal Health). These estrus synchronized ewes were bred by live cover to one ram of like breed (3 Dorsets rams, 1 Southdown ram, and 1 Shropshire ram) as previously described (Reed et al., 2014; Raja et al., 2016; Hoffman et al., 2016a). Date of breeding was considered the day that the ewes were marked by the ram. Pregnancy was confirmed by ultrasound by 30 days of gestation and ewes were moved into individual pens. Ewes were randomly assigned to 1 of 3 diets: 100%, 60% or 140% NRC requirements for energy requirements (total digestible nutrients) and remained on study until they gave birth (Reed et al., 2014). Lambs from control-fed (**CON**), over-fed (**OVER**) and restricted-fed ewes (**RES**) remained with their mother to ensure adequate colostrum intake for up to 24 hours. One lamb from each ewe was removed and euthanized at either 1 day or 3 months of age. Lambs raised until 3 months of age were fed milk replacer (1.7% of BW; Land O'Lakes Animal Milk Product Company; Shoreview, MN) from a bottle until weaning at 60 days of age. They were allowed ad libitum access to water, creep feed (Lamb BT, Blue Seal Feeds; Litchfield, CT), and second cutting hay for the entire three month period (Reed et al., 2014). A total of 33 lambs were used for the study at 1 day (n = 18) and 3 months (n = 15). Information regarding the offspring and gender distribution and selection criterion for offspring have been described in detail (Reed et al., 2014).

Sample Collection and Analysis

Animals were euthanized with an intravenous injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ, USA) containing 390 mg/mL sodium pentobarbital and 50 mg/mL

phenytoin based on BW (0.22 mL/kg), followed by exsanguination, before performing necropsy. At necropsy, femur and tibia bones were collected after removing the wool, skin, and muscles. The femur and tibia from the right leg (1 day, n = 6/treatment; 3 months, n = 4-5/treatment) were used for determining bone mineral content (BMC), bone area and bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA; software version 12·20; GE Lunar Prodigy, Waukesha, WI). Bone marrow MSC were isolated from femur and tibia from the left leg of offspring at 1 day (n = 6/treatment) as previously described (Glynn et al., 2013). Briefly, bones were soaked with PBS and transported to the laboratory. Bones were then rinsed with 70% ethanol and both ends of the bones were removed. In a sterile hood, the interior of the bone was rinsed with α-MEM (Life Technologies, Carlsbad, CA, USA) into a cell culture dish and collected in a Falcon tube (Thermo Fisher Scientific; Waltham, MA, USA) for centrifugation. Bone marrow was centrifuged for three minutes at 1,000 × *g*. Ammonium chloride (Stem Cell Technologies; Vancouver, BC, Canada) was used to lyse red blood cells (RBC). Bone marrow MSC were isolated after filtering the lysed RBC through a 70 μm pore filter. Cells were counted using a hemocytometer and plated at 12 to 15 million cells per cell culture dish (100 x 20 mm; Greiner Bio-One; Monroe, NC, USA) in maintenance media that consisted of α-MEM (Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Atlanta Biologicals; Charlotte, NC, USA), 0.5% Penicillin-Streptomycin and 0.25% Fungizone. Cells were incubated at 37ºC and 5% CO_2 for cell expansion with media changed every two to three days. When the adherent cells were 70 to 80% confluent, they were frozen using 1 mL cell guardian (B-Bridge; Cupertino, CA, USA) per vial in liquid N_2 and were used for subsequent experiments.

Cell proliferation

Cells from lambs (n = 6 per treatment) at 1 day were separately plated in a 96-well plate (1,000 cells/100 μL maintenance media/well; n = 8 wells/lambs) and allowed to adhere for 24 hours, followed by serum deprivation for 24 hours, and then cultured in media with or without 10% FBS for 36 hours. To evaluate cellular proliferation, alamarBlue and 5-bromo-2'-deoxyuridine (BrdU) assays were performed. For alamarBlue assays, cells were rinsed twice (PBS), and a 1:10 dilution of alamarBlue indicator (Life Technologies): α-MEM at 100 μL/well was added. Plates were incubated at 37°C, 5% CO2 and fluorescence was detected at every hour for 24 hours using a Synergy2 plate reader (Biotek; Winooski, VT, USA) at 540/35 × 600/40 nm with a sensitivity of 54. For the BrdU assays, the Cell Proliferation ELISA BrdU (chemiluminescent) kit (Roche Diagnostics; Indianapolis, IN, USA) was used according to the manufacturer's protocol.

Gene expression

Gene expression was determined as previously described (Hoffman et al., 2014; Reed et al., 2014). Briefly, MSC were cultured and seeded at a density of 100,000 cells per well and then allowed to attach and proliferate until they were 70 to 80 % confluent. RNA from MSC was extracted using a Tri-Reagent (Qiagen, Valencia, CA, USA) and Qiagen Mini Kit according to the manufacturer's protocol (Qiagen). Turbo DNA-Free kit (Ambion; Foster City, CA, USA) was used to remove residual genomic DNA. The concentration of RNA was determined using Nano-Drop (Thermo Scientific, Wilmington, DE, USA) and the quality of RNA was determined using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from 300 ng total RNA (10 μL) using OligodT primer (1 μL; Ambion) and master mix containing 5.5 μL of 5x Buffer (Invitrogen), 1.0 μL dNTP (Promega; Madison, WI, USA), 2.0 μL DTT and 0.5

μL Superscript II making total reaction volume of 20 μL. Primers for genes involved in cell proliferation and lineage commitment were designed using Primer3 and NCBI BLAST for *Protein delta homolog 1*, *Msh homeobox 1*, *P2Y purinoceptor 2*, *P2Y purinoceptor 1*, and *P2Y purinoceptor 14*, validated as previously described (Glynn et al., 2013; Hoffman et al., 2014), and synthesized (Integrated DNA Technologies, Coralville, IA, USA; S Table1). Real-time RT-PCR was performed using iTAQ Universal SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and the ABI 7900HT Fast Real-time PCR machine (Applied Biosystems, Foster City, CA). Each reaction included 5μL cDNA, 3 μL of nuclease-free water, 1 μL each of 10 nM forward and reverse primer and 10 μL of Sybergreen (BioRad) making a total volume of 20 μL. Real-time RT-PCR was performed using standard cycling conditions (Stage 1: 50°C for 2 min and 95°C for 10 min, Stage 2: 95°C for 15 s and 60°C for 1 min for 40 cycles, Stage 3: 95°C for 15 s and 60°C for 15 s with a 2% ramp to 95°C for 5 min). $^{\Delta}$Ct values were obtained and used to calculate the $^{\Delta\Delta}$Ct values to determine relative gene expression (Livak and Schmittgen, 2001) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was used as the internal control, as described previously (Glynn et al., 2013).

Mesenchymal stem cell mitochondrial respiration and glycolysis

Glycolytic and mitochondrial stress were evaluated using Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience; North Billerica, MA) according to manufacturer's protocol and previous publications (Qian and Van Houten, 2010; Shum et al., 2016) with the following modifications. Briefly, MSC were plated onto XFe24 cell culture plates at 30,000 cells per well and cultured for 48 hours in standard maintenance media according to protocols published in previous publications (Qian and Van Houten, 2010; Glynn et al., 2013; Shum et al., 2016). The XFe24 sensor cartridges were hydrated with Seahorse Bioscience XFe24 Calibrant (pH 7.4) and

stored at 37 °C for 24 hours (Qian and Van Houten, 2010; Estrada et al., 2012).

For the Glycolysis Stress Test Assay, 2mM glutamine (Sigma-Aldrich; St Louis, MO, USA) was added to Seahorse XF Assay Media (Seahorse Bioscience). The media was warmed to 37^{o}C and pH adjusted to 7.4 with 0.1 N NaOH and filter sterilized. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The wells were rinsed with assay medium two times, and 525 µL of assay medium was added to each well, followed by a 60 minute incubation at 37^{o}C without CO_2. The plate was then loaded into the Seahorse XFe24 Extracellular Flux Analyzer. Standard operating procedure for Glycolysis Stress Test Assay for the machine was followed according to manufacturer's protocol to obtain data.

For the Cell Mito Stress Test Assay, 1 mM pyruvate (Sigma-Aldrich, St Louis, MO), 2 mM glutamine (Sigma-Aldrich), and 10 mM glucose (Sigma-Aldrich) were added to the assay medium, warmed to 37^{o}C and pH adjusted to 7.4 with 0.1 N NaOH. Oligomycin, FCCP, and rotenone/antimycinA were re-suspended with 630 µL, 720 µL, and 540 µL of prepared assay medium, respectively. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The α-MEM maintenance medium was removed with a pipette, and the wells were rinsed twice with assay medium. The wells were then filled with 525 µL of assay medium, and the cell culture plates were incubated for 60 minutes at 37ºC without CO_2. The plate was then loaded into the Seahorse XFe24 Extracellular Flux Analyzer and standard operating procedure for Cell Mito Stress Test Assay for the machine was followed according to manufacturer's protocol.

To account for the variation in final cell density, DNA content in individual wells of XFe24 cell culture plates were quantified using Macharey-Nagel NucleoSpin Tissue kits (Macharey-Nagel Inc, Bethlehem, PA, USA), according to manufacturer's protocol. The

extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) measurements were adjusted for total DNA content in each well.

Cell differentiation and staining

Cells from lambs (n = 6) at 1 day were separately plated at a density of 50,000 cells/well in 6-well (Greiner Bio-One) plates for osteoblast differentiation and 100,000 cells/well for adipocyte differentiation. Cells were induced to differentiate into osteoblasts with addition of media containing 10 nM Dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid (Glynn et al., 2013) or into adipocytes with addition of media containing 10 % rabbit serum, 10 μg/mL Insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 200 μM Indomethacin) (Scott et al., 2011). Cells were stained at day 0 and day 17 of culture in differentiation media with Alizarin red (Sigma-Aldrich) to quantify osteoblast differentiation or Oil Red O (Sigma-Aldrich) to quantify differentiation to adipocytes according to manufacturer's protocol and as previously described (Glynn et al., 2013). For each animal, a total of three images were taken at day 0 and day 17 from three wells of 6-well plates using Axio Cam camera (Zeiss; Jena, Thuringia, Germany) mounted on an Axio Observer microscope (Zeiss) and the area stained was quantified using Image J (NIH) software (Schneider et al., 2012).

Statistical Analysis

For the analysis of proliferation, differentiation, metabolism, and gene expression data PROC MIXED procedure (SAS Inst. Inc, Cary, NC, USA) was used with dietary treatment as the fixed effect. Treatment mean comparisons were performed using LSMEANS statement and PDIFF option. For the proliferation assays, individual wells (n = 8) were averaged to obtain the value for each animal (n = 6/treatment). For the differentiation assays, the difference in the average of staining between day 0 and day 17 was calculated from three images from three

separate wells and averaged together to obtain a value for an individual animal (n = 6/treatment). For the gene expression studies, mRNA from each individual animal was run in triplicate and the average of the $^{\Delta}$Ct values were used for statistical analysis. For the Cell Mito Stress Test Assay and Glycolysis Stress Test Assay (n = 6/treatment) data were obtained by averaging the normalized value obtained from the individual wells to obtain a value per animal. For analyzing bone variables at 1 day or 3 months of age, maternal diet was treated as the fixed effect and a one-way ANACOVA was used, with body weight as a covariate (n = 6/treatment). Outlier analysis was performed and detected outliers were removed. Where appropriate, mean comparisons were made using least square means. Significant differences were determined to be significant at $P \leq 0.05$ or a tendency at $0.05 < P \leq 0.10$.

Results

Bone variables

An effect of poor maternal diet was not observed for femur bone mineral content ($P \geq 0.26$), bone area ($P \geq 0.40$), bone density ($P \geq 0.30$) and bone length ($P \geq 0.22$) at day 1 and at 3 months of age (Table 1). Similar findings were observed for the tibia (data not shown).

Mitochondrial respiration

In all treatment groups, OCR decreased with the injection of oligomycin, increased with the injection of FCCP, and decreased again with the injection of rotenone and antimycin A (Fig 1A). Compared with CON, basal respiration decreased by 29% and 31% in RES and OVER, respectively (127.4 ± 7.5, 90.2 ± 9.8, and 87.5 ± 8.5 ρmol O2/minute/μg DNA; CON, RES, and OVER, respectively; $P \leq 0.007$; Fig 1B). Additionally, ATP production was reduced 30% and 36% in RES and OVER, respectively compared with CON (121.1 ± 6.1, 84.9 ± 11.1, and 77.7 ± 6.4 ρmol O2/minute/μg; CON, RES, and OVER, respectively; $P \leq 0.001$; Fig 1B). Compared

with CON, maximal respiration was reduced 39% and 55% in RES and OVER, respectively (149.29 ± 17.05, 90.64 ± 23.81, 67.93 ± 10.15 ρmol O2/minute/μg DNA; CON, RES, and OVER, respectively; $P \leq 0.03$; Fig 1B). Spare respiratory capacity was reduced in OVER compared with CON, while RES was not different from CON and OVER (21.9 ± 10.8, 0.47 ± 12.3, and -19.56 ± 9.24 ρmol O2/minute/μg DNA; CON, RES, and OVER, respectively; $P \leq 0.06$; Fig 1B). An effect of poor maternal nutrition was not observed for proton leak, non-mitochondrial respiration, coupling efficiency or fold increase of spare respiratory capacity ($P \geq 0.20$; Fig 1B, 1C, and 1D).

Glycolytic stress

The extracellular acidification rate (ECAR) reading increased in all treatment groups after MSC were exposed to glucose, and increased further after the injection of oligomycin and decreased with the injection of 2-DG decreased ECAR in all treatment groups (Fig 2A). An effect of poor maternal nutrition was not observed for any of the variables evaluated for glycolytic stress of MSC, including glycolysis, glycolytic capacity, non-glucolytic acidification rate, and glycolytic reserve capacity ($P \geq 0.18$; Fig 2B and 2C).

Cell proliferation

In presence of serum**,** MSC proliferation was reduced 60 % and 51 %, in OVER and RES, respectively compared with CON (429,780 ± 79,964; 208,902 ± 39,020; 173,498± 35,631 rlu/sec; CON, RES, and OVER, respectively; $P \leq 0.009$; Fig 3). Similarly, in the absence of serum, there was a reduction in MSC proliferation of 39 % and 32 % in the OVER and RES, respectively compared with CON (151,767 ± 5657; 102,756 ± 18,725; 91,847 ± 15,142 rlu/sec; CON, RES, and OVER, respectively; $P \leq 0.04$; Fig 3). A similar magnitude and direction of reduction in proliferation was also observed using alamar blue assay (data not shown).

Cell differentiation

Mesenchymal stem cells were successfully differentiated into adipocyte and osteoblast cells as demonstrated by an 86 % and 94 % increase in area stained, respectively. An effect of poor maternal nutrition on the differentiation of MSC into adipocytes ($P > 0.33$) was not observed in 1 day offspring (Fig 4A) and the representative photographs taken at day 0 and day 17 for CON, RES and OVER are shown (Fig 4B). Also, an impact of poor maternal nutrition on the differentiation of MSC into osteoblasts was not observed ($P > 0.55$) in 1 day offspring (Fig 5A). The representative photographs taken at day 0 and day 17 for CON, RES and OVER are shown (Fig 5B)

Gene expression data

In ovine MSC from 1 day, the expression of P2Y1 tended to be reduced ($P = 0.08$; Table 2) 2.7 fold by the maternal diet in OVER compared with CON. We did not observe an effect ($P \geq 0.14$; Table 2) of poor maternal nutrition on the expression of genes *Pref-1*, *P2Y14*, *P2Y2* or *Msx-2*.

Table 1. Bone variables[a] in CON, RES and OVER lambs at 1 day and 3 months of age.

	Treatment[b,c]				
Item	CON	RES	OVER	SEM	*P*-Value
Mineral content, g					
1 day of age	8.08	7.65	9.75	0.48	0.30
3 months of age	36.69	35.08	39.37	2.34	0.26
Area, cm^2					
1 day of age	20.15	19.34	21.87	0.61	0.40
3 months of age	49.69	47.44	50.10	2.07	0.67
Density, g/cm^2					
1 day of age	0.40	0.39	0.44	0.02	0.30
3 months of age	0.74	0.74	0.78	0.03	0.47
Length, cm					
1 day of age	11.53	11.36	11.90	0.15	0.90
3 months of age	18.10	17.00	18.60	0.41	0.22

[a] Bone mineral content, bone area, and bone density are determined by dual-energy X-ray absorptiometry and bone length was determined using a caliper at 1 day and at 3 months of age for femur and tibia from right hind limb.

[b] Means ± standard error of means (SEM) are reported

[c] Ewe diet was based on the National Research Council (NRC) for energy requirements (total digestible nutrient) for pregnant ewes bearing twins, and began at day 30 ± 0.2 of gestation. Offspring from ewes fed a control (100% NRC, 1 day, n = 6; 3 months, n = 5), restricted (60% NRC, 1 day, n = 6; 3 months, n = 5), or over-fed (140% NRC, 1 day, n = 6; 3 months, n = 4) diet are referred to as CON, RES, and OVER, respectively.

Table 2: Gene expression[a] of key transcription factors involved in proliferation and differentiation of mesenchymal stem cells in CON, RES and OVER offspring at 1 day of age.

	Treatment[b,c]				
Gene	CON	RES	OVER	SEM	*P*-value
Pref-1	1.02	1.12	1.35	0.15	0.25
Msx2	1.21	1.14	0.80	0.40	0.21
P2Y2	1.08	0.98	0.91	0.16	0.75
P2Y1	**1.22**	**0.85**	**0.45**	**0.16**	**0.08**
P2Y14	1.13	0.70	0.68	0.10	0.14

[a] Relative to CON, means ± standard error of means (SEM) are reported

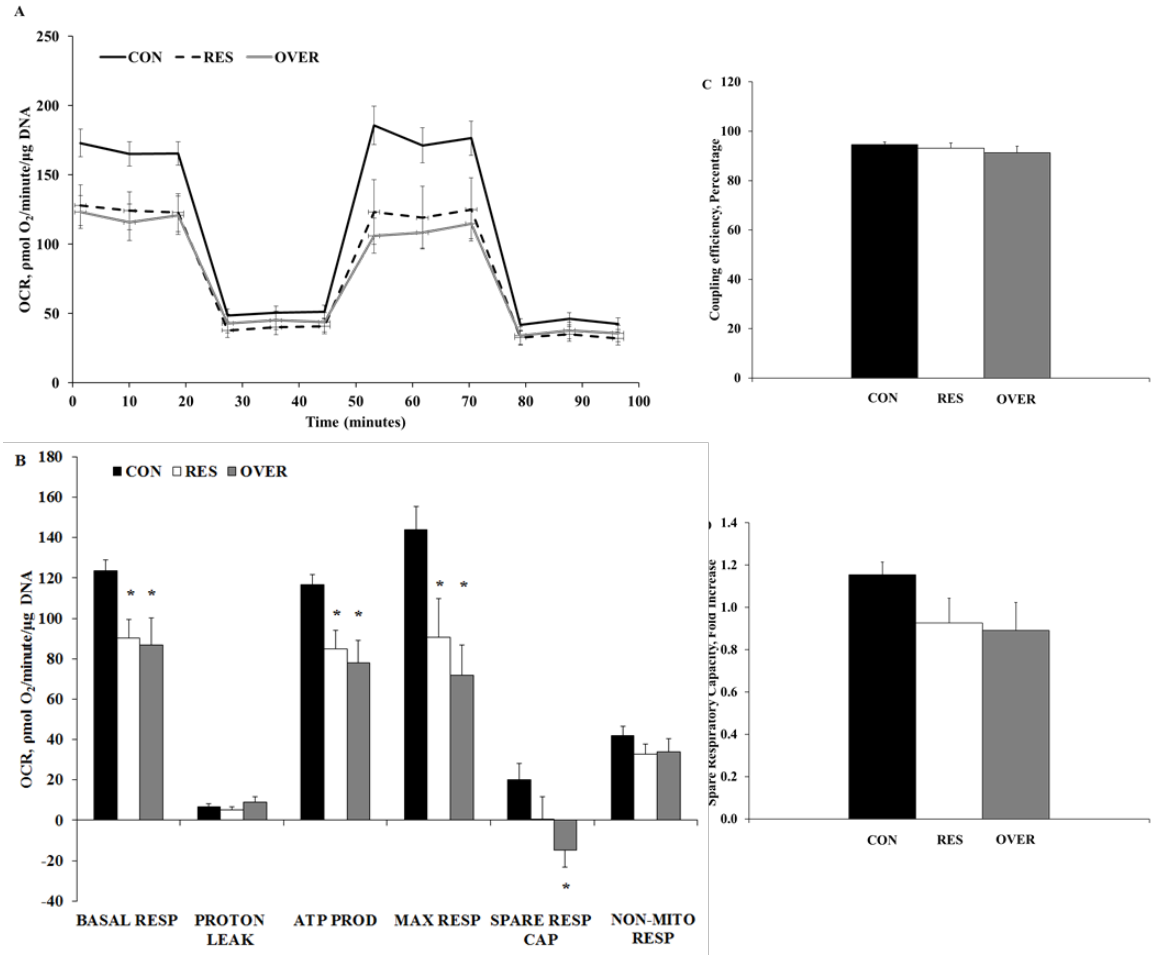
[b] Gene expression was determined by real time RT-PCR for MSC collected from offspring at 1 day of age.

[c] Ewe diet was based on the National Research Council (NRC) for energy requirements (total digestible nutrients) for pregnant ewes bearing twins, and began at day 30 ± 0.2 of gestation. Offspring from ewes fed a control (100% NRC, 1 day, n = 6), restricted (60% NRC, 1 day, n = 6) or over-fed (140% NRC, 1 day, n = 6) diet are referred to as CON, RES, and OVER, respectively.

Supplementary Table 1. Primer sequences

Primers	Forward sequence (5’ – 3’)	Reverse sequence (3’ – 5’)
Protein delta homolog 1	CGG GTT CTC AGG AAA GGA TT	CAG CTG TTG GTC ACG ATC TC
Msh homeobox 1	ACA CAA GAC CAA TCG GAA GC	GGG GAG CAC AGG TCT ATG TG
P2Y purinoceptor 2	CCT CCC TGC CGC TGC TGG TT	TCT GTG GCG GGC TTG GCA TC
P2Y purinoceptor 1	TCC GGA AAA ACA AAA CCA TC	CTC CTC AGA GGC GAA TTG TC
P2Y purinoceptor 14	TGA TCC TGA CCA ATC GGA AT	TGC CCA CAA AGA TGT AGC TG

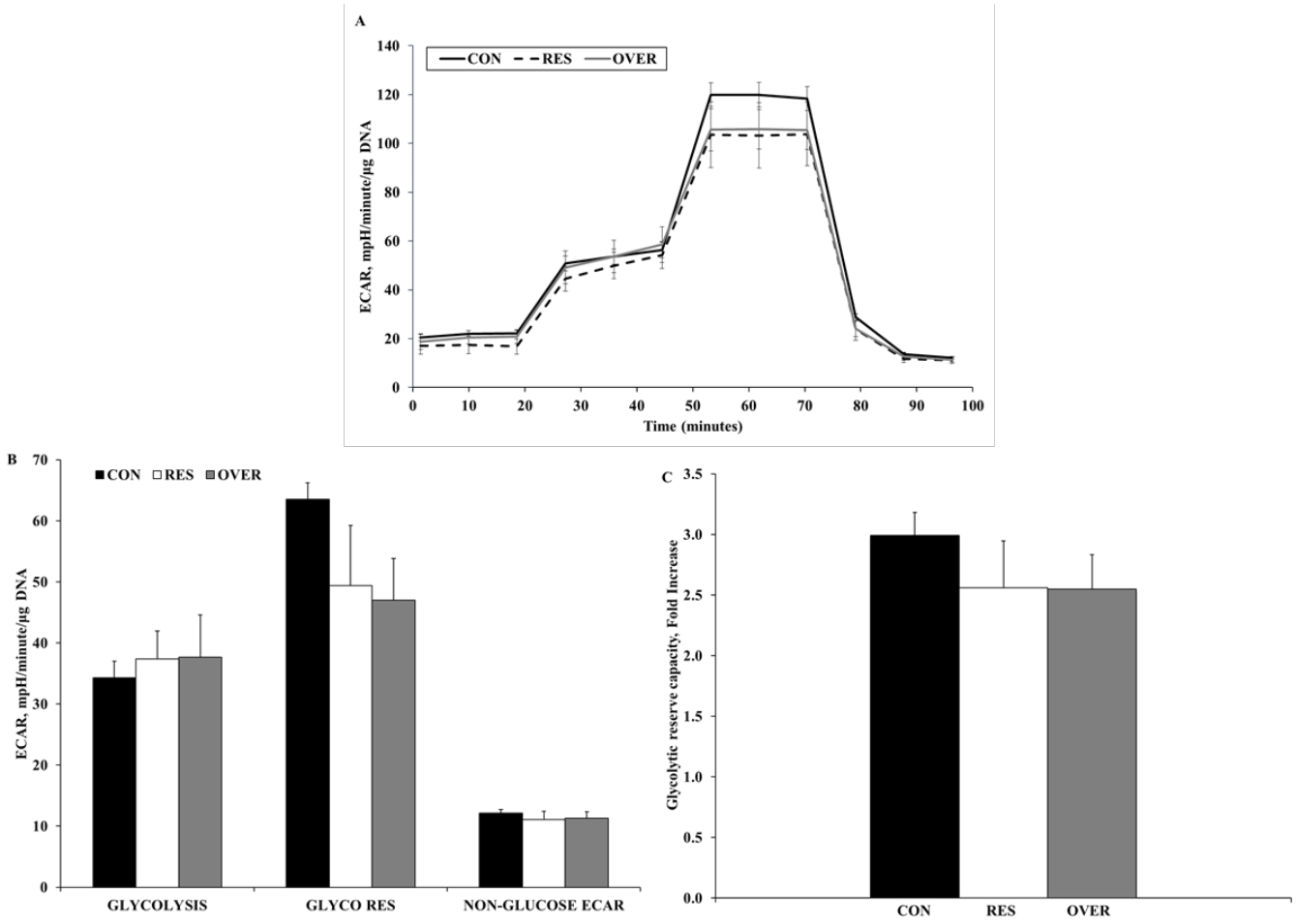
Fig 1. Poor maternal nutrition decreases the oxidative phosphorylation capacity of offspring mesenchymal stem cells.



Mesenchymal stem cells were obtained from the offspring of control-fed (CON, n = 6), restricted-fed (RES, n = 6), and over-fed (OVER, n = 5) ewes at 1 day of age. Data are presented as mean ± standard error. * denotes $P < 0.05$. (A) Oxygen Consumption Rate (OCR), an indicator of oxygen-dependent mitochondrial ATP production, was measured when MSC were subsequently exposed to Oligomycin, FCCP, and Rotenone/Antimycin. (B) Calculation of basal respiration (BASAL RESP), proton leak (PROTON LEAK), ATP production (ATP PROD), maximal respiration (MAX RESP), spare respiratory capacity (SPARE RESP CAP), and non-

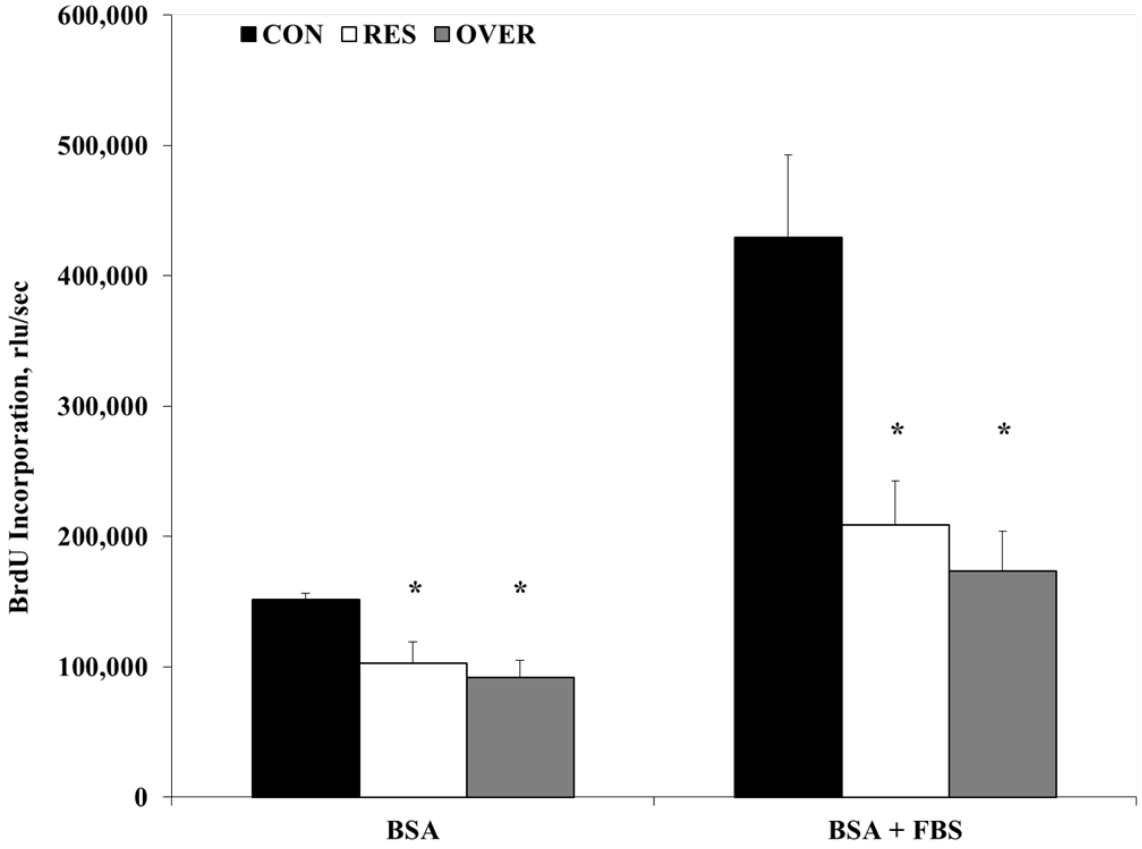
mitochondria-derived respiration (NON-MITO RESP) of MSC, presented as mean ± standard error (C) Coupling efficiency (COUPLING EFF), represented as a percentage ± standard error (D) SPARE RESP CAP presented as a fold increase ± standard error.

Fig 2. Glycolysis Stress Assay for MSC.



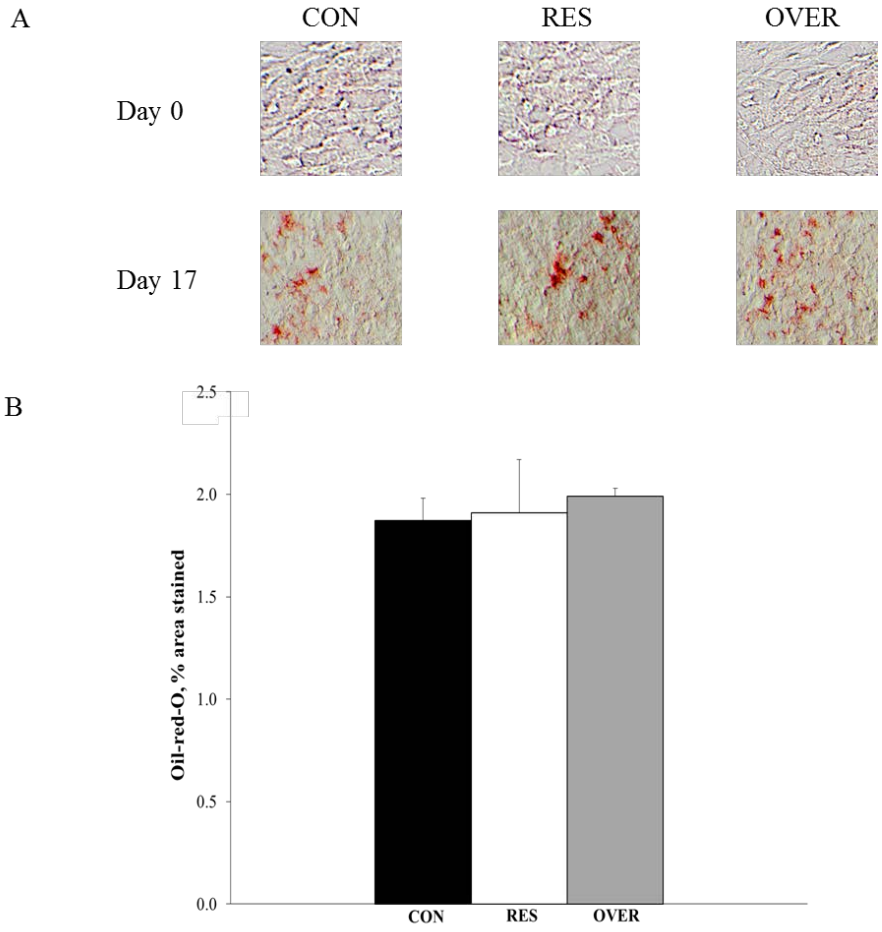
Mesenchymal stem cells were obtained from the offspring of control-fed (CON, n = 6), restricted-fed (RES, n = 6), and over-fed (OVER, n = 5) ewes at 1 day of age. Data are presented as mean ± Standard error. (A) Extracellular Acidification Rate (ECAR), an indication of glycolysis, was measured when MSC were subsequently exposed to glucose, oligomycin, and 2-deoxyglucose. (B) Calculation of basal glycolysis (GLYCOLYSIS), glycolytic reserve (GLYCO RES), and non-glucose-derived ECAR (NON-GLUCOSE ECAR) of MSC presented as mean ± standard error. (C) Glycolytic Reserve Capacity (GLYCO RES CAP) represented as a fold increase, of MSC presented as mean ± standard error.

Fig 3. Poor maternal nutrition reduces proliferation of offspring mesenchymal stem cells.



MSC were obtained from the offspring of control-fed (CON, n = 5), restricted-fed (RES, n = 6), and over-fed (OVER, n = 6) ewes at 1 day of age. Cells were cultured in the absence [bovine serum albumin (BSA)] or presence [BSA + fetal bovine serum (FBS)] of serum and proliferation was determined by 5-bromo-2'-deoxyuridine (BrdU) assay. Data are presented as mean ± standard error. $^{*}P < 0.01$ rfu/s = relative fluorescence units/s.

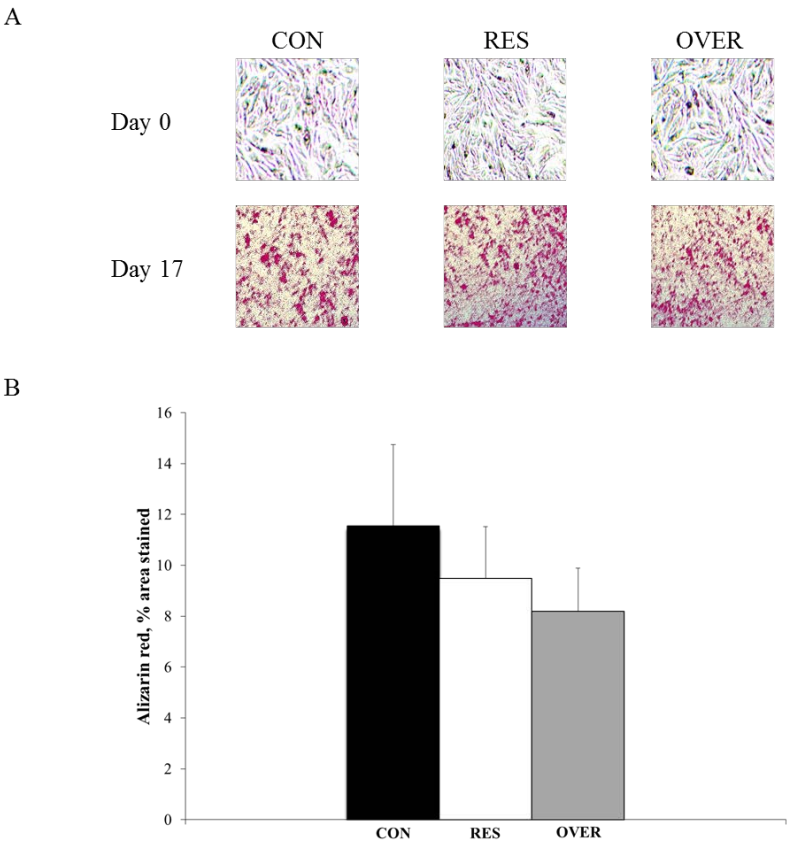
Fig 4 Impact of poor maternal nutrition on differentiation of mesenchymal stem cells into adipocytes



Mesenchymal stem cells were obtained from the offspring of control-fed (CON, n = 6), restricted-fed (RES, n = 6), and over-fed (OVER, n = 6) ewes at 1 day of age. Cells were plated in 6-well plates and differentiation was induced by addition of adipocyte differentiation. ImageJ software was used to quantify staining and data are presented as difference of mean values observed at day 17 and day 0 ± standard error. (A) Representative photographs demonstrating the Oil-red-O staining in CON, RES and OVER at day 0 and day 17 of culture in adipocyte

differentiation are shown (B) Cells were stained with oil-red-o at day 0 and day 17 of culture in adipocyte differentiation media and quantified ($P \geq 0.33$).

Fig 5. Impact of poor maternal nutrition on differentiation of mesenchymal stem cells into osteoblast



Mesenchymal stem cells were obtained from the offspring of control-fed (CON, n = 6), restricted-fed (RES, n = 6), and over-fed (OVER, n = 6) ewes at 1 day of age. Cells were plated in 6-well plates and differentiation was induced by addition of osteoblast differentiation media. ImageJ software was used to quantify staining and data are presented as difference of mean values observed at day 17 and day 0 ± standard error. (A) Representative photographs demonstrating the alizarin red staining in CON, RES and OVER at day 0 and day 17 of culture in osteoblast differentiation are shown (B) Cells were stained with alizarin red at day 0 and day 17 of culture in osteoblast differentiation media and quantified ($P \geq 0.55$).

Discussion

Self-renewal capacity is a key feature of stem cells, which helps to maintain a pool of these cells in the organism (Bianco et al., 2001; Caplan, 2015). Additionally, maintaining these cells is essential for the development and maintenance of various tissues (Ohishi and Schipani, 2010; Caplan, 2015). Specifically, MSC are critical for tissue growth, maintenance, and repair from fetal development through adulthood. Mesenchymal stem cells are key components of the bone marrow niche, which is responsive to nutrient, hormonal, and metabolic changes in the whole body (Reagan and Rosen, 2016). Since these cells were cultured in controlled in vitro conditions, the 50% reduction in proliferation of MSC from both RES and OVER offspring suggests maternal programming of the offspring MSC. Furthermore, our findings of reduced MSC proliferation in lambs are similar to findings in rodents (Oreffo et al., 2003). Mesenchymal stem cells of offspring from mothers fed a low protein diet exhibited reduced proliferation and ability to differentiate into bone forming cells (osteoblasts) when collected from pups 8 weeks of age (Oreffo et al., 2003). This reduced proliferation of MSC may affect the development and maintenance of tissues such as bone, fat, and muscle by reducing the stem cell pool (Du et al., 2011; Devlin and Bouxsein, 2012). We previously reported that maternal nutrient restriction reduced the backfat thickness in the offspring (13), therefore it is possible that alterations to cellular proliferation of MSC may have altered the establishment of this adipose depot. In addition to changes in proliferation, changes to cellular metabolism and function may be mediating some the effects of poor maternal nutrition on offspring growth and development.

Previous studies have found that metabolism is a key determinant of whether a cell proliferates, differentiates, or remains quiescent (Ochocki and Simon, 2013; Shyh-Chang et al., 2013; Hu et al., 2016). A recent study conducted by Lyublinskaya and colleagues found that

reactive oxygen species (ROS) are required for MSC to initiate proliferation (Lyublinskaya et al., 2015). Additionally, reduced levels of basal oxidative phosphorylation will lead to a reduced ROS in MSC (Varum et al., 2011; Shum et al., 2016). Therefore, the reduced level of oxidative phosphorylation in RES and OVER in the current study may be contributing to reduced proliferation in RES and OVER, through a reduction in ROS.

Bone marrow-derived MSC are suited for a low-oxygen niche that promotes the maintenance of the undifferentiated state (Ito and Suda, 2014). However, the MSC used in the culture and assay conditions are no longer in this hypoxic environment and therefore the increased oxygen availability should increase oxygen utilization (Ito and Suda, 2014). mTOR is one such signaling pathway that upregulates metabolism in response to the sensing of increased oxygen and nutrient availability (Ito and Suda, 2014). Therefore, it is expected that cells maintain a greater metabolic state under increased oxygen availability. Under normoxia, the MSC from RES, OVER, and CON all experienced comparable rates of glycolysis, which is likely because oxygen is not involved in glycolysis.

There is an important balance between proliferation (and therefore increased metabolic activity) and stem cell longevity. When proliferation is restricted, stem cell longevity is extended through decreased cellular aging, quiescence, and stem cell exhaustion (Ito and Suda, 2014). Alternately, increased metabolic activity promotes proliferation but can cause cellular injury, especially in stem cells, through ROS and oxidative stress. We found that MSC from RES and OVER had both reduced proliferation and reduced oxidative phosphorylation, compared with CON. Oreffo et al. (2013) also demonstrated that proliferation of MSC was reduced in offspring of protein-restricted ewes. Therefore, it is possible that MSC from RES and OVER are programmed to limit oxidative phosphorylation which limits ROS and preserves stem cell

longevity, as the animal is preparing for birth into an anticipated unfavorable environment. When considering cellular metabolism, the function of the mitochondria in the cell also needs to be taken into consideration.

Mitochondria have an important role in the maintenance and differentiation of stem cells (Shum et al., 2016). During differentiation of MSC into specific cell types, such as osteoblasts or adipocytes, there is a critical shift from glycolytic to aerobic metabolism (Chen et al., 2012). During this shift, there is a need for upregulation of mitochondrial function. In the present study, to gain insight into the effect of maternal nutrition on MSC energy metabolism, we determined mitochondrial respiration and glycolysis capacities of MSC. The reduced basal respiration and ATP production in MSC of both RES and OVER offspring suggest that the MSC have reduced ability to upregulate ATP production during energetic deficits. Additionally, as these findings were observed when cultured in controlled in vitro conditions, they suggest potential programming of these stem cells resulting in impaired differentiation potential. Consistent with reduced ATP production, we observed reduced expression of PGC-1alpha in the muscle of OVER offspring at birth (Hoffman et al., 2016b). This is consistent with the hypothesis that reduced mitochondrial activity in MSC impairs development of tissue, such as muscle. Overall, the changes in the metabolic profile of MSC observed in our study can potentially lead to the development of whole body metabolic dysfunction, and associated metabolic diseases such as insulin resistance, obesity, oxidative stress, and mitochondrial dysfunction in sheep (Wu et al., 2006).

Adequate bone development is important due to its critical role in structural support and framework for the body in addition to its role in protecting soft tissues, maintaining mineral balance, and hematopoiesis (Florencio-Silva et al., 2015). There is evidence that the environment

can alter bone development (14); however previous reports vary depending on species, model, and age. To date most reports of altered bone mass due to poor maternal nutrition during gestation were observed in adult offspring (Fiorotto et al., 1995; Mehta et al., 2002; Ford et al., 2007). In a rat model, maternal protein deprivation during pregnancy modified growth-plate morphology and negatively impacted bone composition, length, and mechanical strength in offspring (Oreffo et al., 2003; Atkinson, 2015). Epidemiological studies have also associated low birth weight with reduced bone mineral content and increased risk of osteoporosis later in life for humans (Cooper et al., 1997; Eastell and Lambert, 2002). The absence of an effect of maternal diet on offspring bone at birth and 3 months of age in the current study may be due to the early time points evaluated. During fetal development, in addition to critical organs such as the brain and heart, bone receives priority for nutrient partitioning and unless a severe restriction occurs, it is unlikely that changes in bone development and mineralization will be detected at this time point. However, due to the loss of bone that naturally occurs in adults and aging humans and animals, it is possible that maternal diet programs the offspring for a similar accelerated bone loss that is observed in adult offspring (Devlin and Bouxsein, 2012). Consistent with the nutrient priority during development, we and others have observed impaired muscle and fat development during the early postnatal period of poorly fed mothers (Fiorotto et al., 1995; Ford et al., 2007; Reed et al., 2014; Hoffman et al., 2016a; Hoffman et al., 2016b). Additionally, restricted feeding during gestation impaired satellite cell function and altered mRNA expression in muscle of offspring (Raja et al., 2016). These findings are consistent with the nutrient priority to bone and organs during early development compared with muscle and adipose. However, it is possible, that poor maternal nutrition may be altering the function of the MSC during gestation and therefore impacting the role of these cells in establishing key tissues, such as the muscle and

adipose. To better understand this potential involvement, we are currently evaluating the effects of poor maternal nutrition on MSC during fetal development.

Based on the knowledge that many tissues of mesenchyme origin are also developed and maintained during postnatal growth through the pool of MSC, we further investigated the effects of maternal diet on the function of offspring MSC. Specifically, based on the recent reports that changes in gene expression and maternal diet can alter the lineage commitment of MSC (Woo et al., 2011; Devlin and Bouxsein, 2012), we determined if maternal diet altered the ability of MSC to differentiate into adipocytes and osteoblasts. Consistent with bone phenotype, we did not observe an effect of diet on differentiation into osteoblasts or adipocytes. The absence of an effect of maternal diet on the differentiation of MSC might be due to the early time point at which the samples were collected or variation from the methods used for the estimation of differentiation. Further studies are warranted at different time points to evaluate the effect of poor maternal nutrition on differentiation ability of MSC.

Several key transcription factors and regulatory genes determine the ability of MSC to proliferate and differentiate of MSC (Sawada et al., 2006). Of particular interest are the purinergic receptors, which have critical roles in committing MSC to differentiate into adipocyte or osteoblasts lineages (Kaebisch et al., 2014; Kaebisch et al., 2015). *Purinergic receptor 2Y* is involved in the proliferation of MSC, as well as the differentiation and migration of MSC (Kaebisch et al., 2014). Therefore, the reduced expression of *Purinergic receptor 2Y 1* in the OVER group could be a potential mechanism contributing to the reduced proliferation. However, further studies to explore its role in MSC response to maternal diet, including changes in protein expression, are needed. Additional analysis is needed to evaluate global changes in gene expression to further elucidate the mechanisms by which maternal diet alters offspring MSC

function.

In conclusion, the findings in the current study demonstrated the programming effects of poor maternal nutrition during gestation on offspring stem cell function and metabolism. Furthermore, this study demonstrates that alterations to the cellular bioenergetics of MSC as a possible mechanism for the reduced MSC proliferation and more importantly previously reported impaired muscle, bone, and adipose development. The molecular mechanisms by which these changes occur in MSC are currently unknown and warrant further investigation.

CHAPTER 2: FETAL AND ORGAN DEVELOPMENT AT GESTATIONAL DAYS 45, 90, 135 AND AT BIRTH OF LAMBS EXPOSED TO UNDER- OR OVER-NUTRITION DURING GESTATION

Pillai, S. M., A. K. Jones, M. L. Hoffman, K. K. McFadden, S. A. Zinn, S. A. Reed, and K. E. Govoni. 2016a. Fetal and organ development at gestational days 45, 90, 135 and at birth of lambs exposed to under- or over-nutrition during gestation. Translational Animal Science. doi: 10.2527/tas2016.0002

INTRODUCTION

Variations in feed and forage quality and availability can result in periods of sub-optimal nutrition for livestock species. This is problematic as poor maternal nutrition during gestation has immediate and long-lasting consequences on production efficiency and health of offspring, including reductions in birth weight, pre-weaning survival, postnatal growth rate, feed utilization, carcass quality and lifespan (Godfrey and Barker, 2001; Wu et al., 2006; Caton and Hess, 2010; Ford and Long, 2011; Du et al., 2013; Reed et al., 2014). Maternal programming is defined as alterations to the intrauterine environment that affect the growth and development of the fetus, resulting in changes in offspring growth, metabolism and organogenesis (Wu et al., 2006). Organogenesis primarily occurs during gestation, making it especially vulnerable to the effects poor maternal nutrition. As a result, multiple organ systems can potentially be affected; thus, predisposing offspring to metabolic and endocrine disorders (Hyatt et al., 2008; Benz and Amann, 2010; Tarry-Adkins et al., 2013).

We previously demonstrated that both restricted- or over-feeding during gestation can alter lamb growth rates, muscle and adipose composition, and organ size at early postnatal time points (Godfrey and Barker, 2001; Hoffman et al., 2014; Reed et al., 2014; Raja et al., 2016; Hoffman et al., 2016a). Although others have evaluated the effects of maternal diet at specific time points of gestation, most were targeted at 1 to 2 time points and one dietary treatment

(Godfrey and Barker, 2001; Vonnahme et al., 2003; Wu et al., 2006). Therefore, to better understand when poor maternal nutrition affects growth and organ development during gestation, our objective was to develop a model that would evaluate the effects of poor maternal nutrition on fetal and organ growth at three gestational and one early postnatal time point, with the ability to compare control, restricted and over feeding in one experiment. We hypothesized that maternal restricted- and over-feeding during gestation would negatively impact offspring growth and organ development at specific stages of gestation and at birth.

MATERIALS AND METHODS

All animal procedures were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee (A13-059).

Multiparous Western White-faced ewes (n = 82), 3 years or older were estrus synchronized and bred as previously described (Jones et al., 2016). Briefly, a controlled intravaginal drug release device (CIDR; Easi-Breed CIDR Sheep Insert, Zoetis, Florham, NJ) was inserted intravaginally into each ewe, removed after 12 d and then ewes received a single injection of $PGF_{2\alpha}$ i.m. [Lutalyse, 5 mg/mL; Zoetis; (Knights et al., 2001)]. Ewes were group housed with one of four related Dorset rams, obtained from a closed flock, for breeding. Day 0 of pregnancy was considered the day that the ewe received a rump mark with no subsequent remarking. Twenty days after mating, ewes were moved to individual pens (3 x 1 m; with visual but not physical contact with other ewes) and each ewe was fed individually for the duration of the experiment. At d 20, over a 7 d period, ewes were transitioned onto a complete pelleted feed at 100 % NRC. Eighty-two ewes were confirmed pregnant by trans-abdominal ultrasound on d 28.5 ± 0.4 (Jones et al., 2016) and were randomly assigned into a 3 x 4 factorial arrangement of treatment structure at d 30 of gestation with main effects of diet (3) and time point of gestation

(4; n = 5 to 7 ewes per treatment combination; Fig. 1).

At d 30.2 ± 0.2 of gestation, pregnant ewes were fed either a control (100%; n = 27), restricted (60%; n = 28) or over (140%; n = 27) diet based on the NRC requirement for TDN (National Research Council, 1985). Treatment diets (Central Connecticut Farmer's COOP, Manchester, CT; Table 1) of 60, 100 and 140 % NRC were achieved by offering different quantities of the complete pelleted feed based on ewe BW as previously described (Hoffman et al., 2014; Reed et al., 2014; Hoffman et al., 2016a; 2016b). Nutrient analyses were completed by Dairy One, Inc. (Ithaca, NY; Table 1) for each new grain delivery (n = 3). Fresh water and blocks containing sodium chloride only were available to ewes ad libitum. Ewes were weighed and BCS (Russel, 1984) was independently evaluated by two trained observers at the beginning of dietary treatment, and weekly thereafter. Ewes remained on their diets until they were euthanized at one of the three gestational time points or until parturition.

At d 45, d 90 or d 135 of gestation, ewes (n = 5 to 7 per treatment combination) were weighed and euthanized with an i.v. injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ) containing 390 mg/mL sodium pentobarbital and 50 mg/mL phenytoin based on BW, followed by exsanguination as previously described (Reed et al., 2014). Subsequently, a hysterectomy was performed to remove the uterus and fetuses for tissue collection. A fourth group of ewes was allowed to undergo parturition (birth; n = 5 to 7 per dietary treatment). Lambs at birth were allowed to nurse for up to 24 h before euthanasia to ensure adequate colostrum intake. If a ewe did not produce colostrum, lambs were offered manufactured colostrum replacer (Four ewes total: two twin control litters and two twin restricted litters; DuMor Blue Ribbon Multi-Species Colostrum Supplement; Brentwood, TN). Within 24 h of parturition, lambs were weighed and euthanized as described above for tissue collection. Offspring born to control-,

restricted-, or over-fed ewes are referred to as CON, RES and OVER, respectively, hereafter. Four ewes did not complete the study due to reasons unrelated to the experiment; thus 78 ewes and their respective offspring were available for fetal and organ measurements. Five offspring were not viable (2 mummified and 3 still born) and were not included for analysis, for a total of 147 offspring from the 78 ewes that were included in the analysis. There were missing data at d 45 for offspring BW (CON Singleton: n = 1 fetus) and at d 90 for offspring BW (CON Singleton: n = 1 fetus; RES Triplet: n = 1 fetus).

At d 45, identification of fetuses as male or female by visual inspection was not possible. Therefore, male fetuses were identified as those individuals that expressed the sex-determining region Y (SRY) gene using endpoint PCR. Briefly, tails were collected from d 45 fetuses at necropsy, snap frozen in liquid nitrogen, and stored at -20° C. DNA was isolated as previously described (Truett et al., 2000). Primers for the sex-determining region Y (SRY) gene (Forward: 5'-AACGAAGACGAAAGGTGGCT-3' Reverse: 3' –TCCTCAAAGAATGGGCGCTT-5') were designed using the ovine reference sequence (AY604733.1), NCBI-Primer blast, and UCSC In-silico PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Forward: 5'-GGCGTGAACCACGAGAAGTATAA-3' and Reverse: 3'-CCTCCACGATGCCAAAGTG-5') was used as the positive control (Buza et al., 2009). Endpoint PCR was performed as previously described (Hoffman et al., 2014). The sex of offspring and litter size distribution for all animals included for these analyses are reported in Table 2.

Before sample collection, offspring BW was recorded. To obtain fetal organs, a mid-ventral incision extending from the thoracic cavity to the lower abdominal cavity was made. Organs [liver, heart, kidneys (both combined), adrenal glands (both combined) and perirenal fat (both sides)] were excised from each offspring and weighed. Organ measurements [heart length

and width, kidney length, and rib width (averaged for the 5th, 6th, and 7th rib)] were obtained using a handheld electronic digital caliper at all time points. Loin eye area (LEA) of the LM was measured between the 12th and 13th rib using a grid specific to pork and lamb (Iowa State University Extension and Outreach, Ames, IA). At d 45 of gestation, the adrenal glands, and perirenal fat were not detectable. The LEA was only measurable at d 135 and birth due to the small size at earlier time points.

All data were analyzed using PROC MIXED (SAS Institute Inc, Cary, NC; version 9.4). Ewe BW and BCS data were analyzed as a completely randomized design, with fixed effects of diet, day of gestation, litter size, and their interactions. Sire had no effect on the fetal variables presented ($P \geq 0.12$) and therefore was removed from the model. Offspring data were analyzed as a split-plot design. In the whole plot, ewes were randomly assigned into the 3 x 4 factorial arrangement of treatments of diet (3) and day of gestation (4). In the subplot, offspring were assigned to a 3 x 4 x 3 x 2 factorial arrangement of treatments, including maternal diet (3), day of gestation (4), litter size (3) and sex (2), respectively and listed as the fixed effects with all interactions. A random statement of ewe by diet by day of gestation was included to define the whole plot. Organ data were expressed as a percent of BW to understand organ growth relative to BW, and all data are presented as least square means ± SE. Significance was considered at $P \leq 0.05$. Comparisons of pairwise incidence for the maternal diet by day of gestation were conducted if significant using the LSMEANS statement with PDIFF option. In the absence of a maternal diet by day of gestation interaction, main effects are discussed.

RESULTS

Significant 3-way interactions of litter size by maternal diet by day of gestation were observed for offspring BW ($P = 0.001$), and perirenal fat expressed as a percent of BW ($P =$

0.02; Supplementary Table 1). No 3-way interactions of litter size by maternal diet by day of gestation were observed for weights of organs expressed as a percent of BW ($P \geq 0.09$). Thus, in the interest of how maternal diet affects the offspring at specific stages of gestation, only the main effects and interaction of maternal diet and day of gestation are reported herein.

A significant interaction of maternal diet by day of gestation was observed for offspring BW ($P = 0.03$; Table 4) and heart girth circumference ($P = 0.009$; Table 4). No differences between maternal diets were observed for fetal BW at d 45, 90 or 135 of gestation ($P \geq 0.24$). However, at birth, RES offspring weighed 18.4% less than CON and 13.1% less than OVER (Table 4; $P \leq 0.04$)

Due to the differences observed in offspring BW, all organs were expressed as a percent of BW (actual organ weight data are provided in Supplementary Table 2). When expressed as percent of BW, an interaction of maternal diet by day of gestation was observed for weight of offspring kidneys ($P = 0.02$), liver ($P = 0.01$), and perirenal fat ($P = 0.002$; Table 3). At d 45 of gestation, the kidneys of OVER offspring were 35.6% larger than CON and 26.0% larger than RES ($P \leq 0.04$). However, at the d 90, d 135 and birth time points no differences in fetal kidney weights were observed between maternal diets ($P \geq 0.60$; Table 3). At d 45 of gestation, the liver of RES offspring was 13.0% larger than CON and 15.6% larger than OVER offspring ($P \leq 0.002$). However, these differences were not maintained in the liver between CON, RES and OVER at d 90, d 135 or birth time points ($P \geq 0.07$). Perirenal fat was not detectable at d 45 of gestation, and no differences in perirenal fat were observed in offspring at d 90 of gestation ($P \geq 0.51$). However, at d 135 of gestation, OVER offspring had 26.3% more perirenal fat than CON and RES lambs ($P \leq 0.03$). At birth, RES offspring had 26.4% more perirenal fat than CON and 40% more perirenal fat than OVER lambs ($P \leq 0.04$). No interactions of maternal diet by day of

gestation were observed for weight of the adrenal glands or heart as a percent BW ($P \geq 0.23$; Table 3). A main effect of maternal diet was observed for heart length, such that the hearts of RES and OVER offspring were 8.1% and 11.1% shorter than CON, respectively, regardless of the stage of gestation (55.9 ± 3.6, 51.7 ± 2.7, 50.3 ± 2.8, mm; CON, RES, OVER, respectively; $P \leq 0.05$). A main effect of gestation was observed for all organ variables ($P < 0.0001$), such that organ weight as a percent of BW decreased (Table 4) but average organ size increased as gestation advanced ($P < 0.0001$; Supplementary Table 2). The width of the heart and ribs of offspring, and length of the heart and kidneys of offspring increased as gestation advanced ($P < 0.0001$). The loin eye area was measurable in offspring only at d 135 and birth, and no main effect of gestation, maternal diet, or interaction was observed ($P \geq 0.32$; Table 3).

DISCUSSION

To maximize production efficiency, animals need to grow quickly and produce a carcass that yields more muscle than fat. However, the growth and development of the offspring can be negatively influenced by environmental stressors to which the dam is exposed during gestation, such as poor maternal nutrition. In livestock species, both restricted- and over-feeding during gestation results in reduced growth rates, reduced muscle fiber size, and increased adiposity of the offspring (Redmer et al., 2004; Wu et al., 2006; Du et al., 2013). Although both under- and over-feeding often lead to similar phenotypic changes in offspring, many studies only focus on one model of poor maternal nutrition such as under- (Vonnahme et al., 2003; Daniel et al., 2007; Ford et al., 2007; Ge et al., 2013) or over-feeding (Zhu et al., 2008; Tong et al., 2009; Caton et al., 2009a; Long et al., 2010; Yan et al., 2011). We have developed a unique model that includes both restricted- and over-feeding of the ewe beginning at early gestation (Hoffman et al., 2014; Reed et al., 2014; Jones et al., 2016; Raja et al., 2016; Hoffman et al., 2016a; 2016b). This

allows for direct comparisons between offspring from control-, restricted- and over-fed mothers. These comparisons are important because as we previously reported, although negative effects on muscle and metabolism are similar, mechanisms contributing to these phenotypes are different in offspring from restricted and over-fed mothers (Hoffman et al., 2016b). Proper organ and tissue growth during fetal development is essential for optimal health and growth during postnatal life. In sheep, fetal organogenesis is largely complete during early gestation, yet only 10% of fetal growth has occurred by d 90 of gestation (Vonnahme et al., 2003; Redmer et al., 2004; Caton and Hess, 2010). Any delay or altered growth during gestation can have negative effects on production. The current model includes key time points during early (d 45), mid (d 90) and late (d 135) gestation and immediately following parturition, allowing us to target specific stages of development. By developing a model of both maternal under- and over-feeding and 4 developmental time points for offspring analysis in one study, we have the ability to make treatment by time comparisons to further understand the impact of maternal nutrition on offspring growth and development over time.

In ruminants, the majority of fetal growth occurs during the last third of gestation while organogenesis occurs during the first third of gestation (Redmer et al., 2004; Vonnahme, 2007; Caton et al., 2009b). Therefore, the effects of maternal nutrition on fetal size and organ development may be time dependent. In the current study, differences in offspring body size were observed at birth whereas differences in fetal organ mass were primarily observed at d 45 and 90 of gestation. The lack of BW difference observed during early- and mid-gestation is consistent with previous studies in which reduced offspring BW in offspring of both restricted- (Wallace et al., 2015; Hoffman et al., 2016a) and over-fed (Caton et al., 2009b; Hoffman et al., 2014) ewes were observed at d 130 of gestation, but not at d 50 or 90. It should be noted that

offspring BW in response to maternal diet is variable with many reporting no effect on BW (Long et al., 2009; Peel et al., 2012; Kleemann et al., 2015), but significant changes in body composition, growth or metabolism.

Although BW differences were not observed until birth, changes in body composition occur earlier, which can affect overall performance and health of offspring later in life. In support of the thrifty phenotype hypothesis (Godfrey and Barker, 2001), increased perirenal fat was observed in both RES and OVER offspring by d 135 of gestation. The increase in perirenal fat may be indicative of offspring preparing for a suboptimal postnatal environment (Godfrey and Barker, 2001). In particular, the RES offspring are programmed to survive on minimal nutrients at birth. However, in the OVER, this may be due to excess nutrients during gestation and altered glucose and/or insulin metabolism. Perirenal fat during early prenatal life is vital for thermogenesis and therefore has a key role in offspring survival. Therefore, it is likely that accretion of perirenal fat is particularly sensitive to maternal nutrient intake before or during mid-gestation, with the resulting phenotype observed in late-gestation or postnatally. In support of this, when ewes were under-fed between d 28 and d 78 of gestation, lambs reared until 250 d of age exhibited increased BW gain, adiposity, and fat to lean ratio (Ford et al., 2007) indicating that maternal under-feeding from early to mid-gestation contributed to postnatal adiposity. Similarly, maternal obesity can lead to increased fat accumulation in adult offspring (Long et al., 2015). Increased adiposity can negatively affect the metabolic health of the animal and compromise carcass quality later in life (Ford et al., 2007; Du et al., 2013; 2015).

After only 15 d of dietary intervention, both restricted- and over-feeding of ewes altered the growth patterns of the fetal liver and kidney, respectively during the first third of gestation. Ovine hepatogenesis occurs as early as d 30 of gestation, and typically the liver occupies less

space in the abdomen with advancing gestation (Douart et al., 2015). Although the liver size increased for all treatment groups over time and were similar during late gestation, the liver of RES offspring was larger at d 45. Similarly, Meyer et al. reported increased liver size, when adjusted for BW, in offspring of restricted-fed mothers (Meyer et al., 2013). Altered development of the liver as a result of poor maternal nutrition can result in hepatic triglyceride accumulation, inflammation and insulin resistance leading to increased visceral adiposity (Kabir et al., 2005). Therefore, early changes in liver development may be linked to the increased perirenal fat observed at birth, indicative that maternal nutrition affects multiple organ systems in an inter-dependent manner. Further studies are needed in the sheep model to determine if the changes in liver development are linked to changes in metabolism and/or postnatal growth of the offspring.

Maternal over-feeding caused early acceleration of kidney growth in offspring at d 45 of gestation. This is consistent with Jackson et al. (2012), who reported that over-feeding during gestation in rats altered development and functions of kidney in offspring exposed to over-nutrition during gestation (Jackson et al., 2012). In addition, in humans and sheep exposure of the fetus to suboptimal maternal diet during gestation has been linked to poor postnatal development and pathological conditions of kidneys (Moritz and Wintour, 1999; Luyckx and Brenner, 2015). For example, decreased protein nutrition in rats reduces the number of nephrons in kidneys and affects renin-angiotensin system, which may predispose offspring to adult diseases, such as hypertension (Woods et al., 2001). The absence of an effect of maternal diet observed after d 45 of gestation is likely due to nutrient partitioning towards the liver and kidney, because ruminants rely on the liver for gluconeogenesis and regulation of the somatotropic axis, and the kidneys for phosphate and calcium balance (Hyatt et al., 2007; 2008; Benz and Amann,

2010; Douart et al., 2015; Luyckx and Brenner, 2015).

CONCLUSION

There is a need to increase the efficiency of production to meet increasing global demand for food, including protein sources, while developing and maintaining a sustainable production system. Nutrient availability to the dam has direct effects on fetal development and permanent effects on offspring health and physiology later in life. The effects of poor maternal nutrition can also impact meat quality and quantity. By developing a model to test the effects of poor maternal nutrition, including both restricted- and over-feeding models, during gestation on offspring growth and health, it will be possible to identify methods to manage the negative outcomes and improve production efficiency. This is important based on recent findings that although changes in muscle were similar in RES and OVER offspring, changes in gene expression were different, suggesting the involvement of different mechanisms (Hoffman et al., 2016b). The current study has developed a model to evaluate key stages of fetal development in poorly fed ewes that identified that poor maternal diet can have negative effects within 2 weeks of dietary changes and as early as d 45 of gestation with effects persisting until birth. In conclusion, we determined that both maternal restricted- and over-feeding during gestation differentially alter organogenesis of the liver and kidneys during early gestation, and body size and composition during late gestation and at parturition. Further investigation into the tissue structure and molecular regulation of each organ at each stage of gestation is necessary to identify mechanisms by which poor maternal nutrition predisposes postnatal organ dysfunction, even in the absence of alterations in mass.

Figure 1

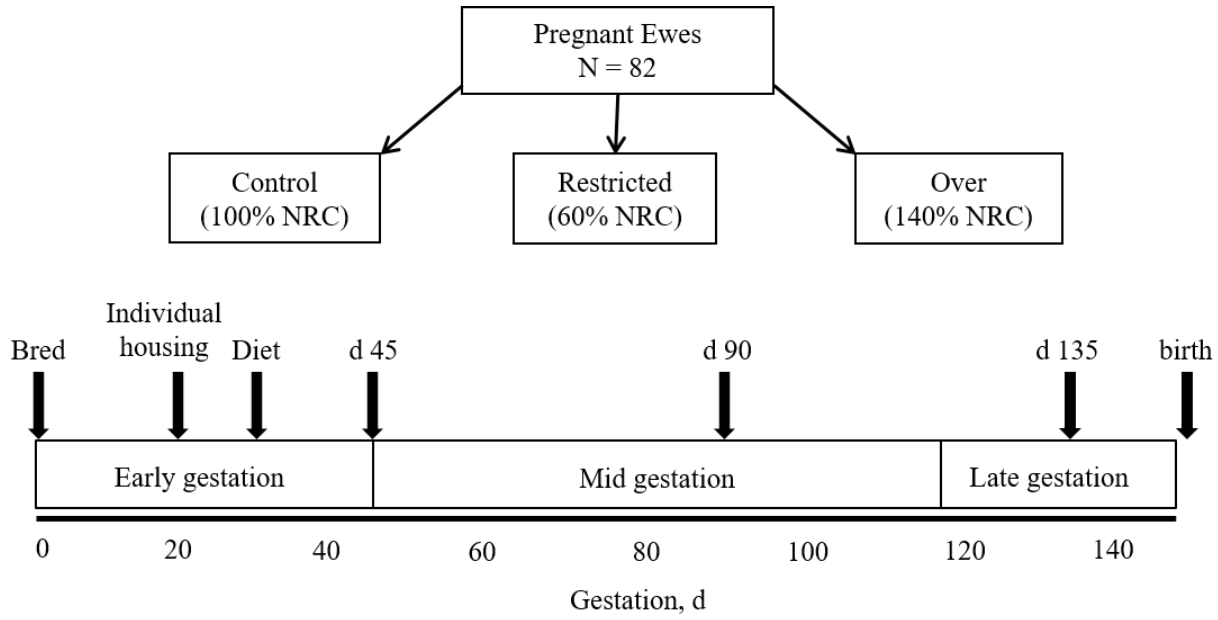


Figure 1. Experimental design. To evaluate the effects of restricted- or over-feeding on offspring development during gestation, pregnant ewes (n = 82) were housed in individual pens on d 20 of gestation (Individual housing) and randomly assigned into a 3 x 4 factorial arrangement of treatments which began at d 30 of gestation (Diet). The treatment structure included three dietary levels (Control: 100% TDN; Restricted: 60% TDN; Over: 140% TDN) and four time points during gestation (n = 5 to 7 ewes per treatment combination). At d 45, 90 or 135 of gestation, ewes were euthanized, and the fetus(es) were removed for necropsy and tissue analysis. A fourth group of ewes was allowed to undergo parturition (birth), after which lambs were necropsied within 24 h of parturition.

Table 1. Formulation and chemical composition of ewe diet (as-fed basis)

Item	Diet Composition
Ingredient[1], %	
Soybean hulls	42.86
Alfalfa meal, 17%	20.00
Ground beet pulp	16.67
Corn meal, fine	16.20
Molasses	3.00
Monoamonium phosphate	0.65
Sheep premix	0.42
Salt	0.21
Nutrient Analysis[2]	
DE, Mcal/kg	3.28
ME, Mcal/kg	2.86
Moisture, %	10.23
DM, %	89.80
CP, %	11.57
Adjusted CP, %	11.57
ADF, %	28.07
NDF, %	40.40
TDN, %	65.67
$NE_{L,}$ Mcal/kg	1.53
$NE_{M,}$ Mcal/kg	1.53
$NE_{G,}$ Mcal/kg	0.98
Ca, %	0.89
P, %	0.34
Mg, %	0.21
K, %	1.09
Na, %	0.11
Fe, mg/kg	381.00
Zn, mg/kg	64.00
Cu, mg/kg	7.00
Mn, mg/kg	56.33
Mo, mg/kg	1.33
S, %	0.21

[1] Feed was formulated by Central Connecticut Co-Op (Manchester, CT)

[2] Nutrient analyses were performed by Dairy One, Inc. (Ithaca, NY; average analysis of three separate grain deliveries for entire experiment).

Table 2. Number of ewes assigned per diet and day of gestation that completed study[1] and the resulting number of offspring acquired for analysis

	Treatment[2]			
Item	Control	Restricted	Over	Sum
Pregnant ewes	25	27	26	78
d 45	7	7	7	21
d 90	7	7	6	20
d 135	6	6	7	19
Birth	5	7	6	18
Total offspring	47	51	49	147
d 45	12	12	15	39
d 90	14	14	11	39
d 135	11	12	11	34
Birth	10	13	12	35
Singleton offspring	7	6	6	19
d 45	3	3	1	7
d 90	2	2	2	6
d 135	1	1	2	4
Birth	1	0	1	2
Twin offspring	23	32	31	86
d 45	6	6	8	20
d 90	6	6	6	18
d 135	7	8	9	24
Birth	4	12	8	24
Triplet offspring	17	13	12	42
d 45	3	3	6	12
d 90	6	6	3	15
d 135	3	3	0	6
Birth	5	1	3	9
Male:Female offspring ratio	31:16	28:23	21:28	80:67
d 45	12:0	9:3	11:4	32:7
d 90	8:6	8:6	3:8	19:20
d 135	7:4	8:4	3:8	18:16
Birth	4:6	3:10	4:8	11:24

[1] Four ewes did not complete study due to reasons unrelated to the experiment. These ewes and their offspring are not included in this table or any of the data analyses (Control, Birth n = 2; Restricted, d 135 n = 1; Over-fed, Birth n = 1). Beginning at d 30 of gestation, pregnant ewes were randomly assigned to a control-fed (100%), restricted-fed (60%) or over-fed (140%) diet based on NRC TDN requirements. Ewes were euthanized at d 45, 90 or 135 of gestation and a hysterectomy was performed to acquire the fetus(es), or ewes were allowed to undergo parturition in which lambs were necropsied within 24 h of birth.

Table 3. Effects of maternal diet and day of gestation on fetal organ variables

	Treatment[1, 2]				*P*-value
Fetal variable[3]	CON	RES	OVER	SEM	Maternal diet by day of gestation
Adrenal gland wt, [4] %BW					0.0991
d 45	nd[5]	nd	nd	nd	
d 90	0.028	0.030	0.031	0.0004	
d 135	0.012	0.010	0.011	0.0009	
Birth	0.020	0.024	0.021	0.0017	
Heart wt, %BW					0.2309
d 45	1.06	1.22	1.31	0.07	
d 90	0.85	0.79	0.83	0.02	
d 135	0.68	0.50	0.66	0.02	
Birth	0.77	0.87	0.82	0.02	
Heart length, mm					0.5226
d 45	nd	nd	nd	nd	
d 90	31.6	30.4	30.9	3.20	
d 135	68.3	56.1	60.2	11.03	
Birth	67.7	64.2	63.9	6.24	
Heart width, mm					0.9839
d 45	6.3	5.8	6.2	0.60	
d 90	23.2	22.5	22.2	2.77	
d 135	49.9	43.8	42.9	4.79	
Birth	49.6	47.9	45.9	4.03	
Kidney wt, [6] %BW					0.0213
d 45	0.67^{ad}	0.77^{ace}	1.04^{b}	0.07	
d 90	0.96^{bc}	0.97^{bc}	0.99^{b}	0.02	
d 135	0.54^{ad}	0.47^{d}	0.59^{de}	0.02	
Birth	0.59^{d}	0.57^{de}	0.55^{de}	0.02	
Kidney length, mm					0.9667
d 45	nd	nd	nd	nd	
d 90	22.3	22.0	23.4	1.45	
d 135	43.2	38.6	39.7	6.35	
Birth	40.1	37.7	39.0	3.63	
Loin eye area, [7] mm^2					0.9813
d 45	nd	nd	nd	nd	
d 90	nd	nd	nd	nd	
d 135	266.5	282.8	274.8	24.6	
Birth	316.7	254.8	280.7	25.2	
Liver wt, %BW					0.0114
d 45	6.7^{a}	7.7^{b}	6.5^{ad}	0.21	
d 90	5.5^{c}	5.4^{c}	5.8^{cd}	0.09	
d 135	2.8^{e}	2.1^{e}	2.8^{e}	0.12	
Birth	2.2^{e}	2.3^{e}	2.3^{e}	0.06	
Perirenal fat wt, [8] %BW					0.0017
d 45	nd	nd	nd	nd	

d 90	0.44[ab]	0.40[a]	0.44[ab]	0.02	
d 135	0.42[ac]	0.42[ac]	0.57[b]	0.02	
Birth	0.39[a]	0.53[bc]	0.35[a]	0.03	
Rib width, mm					0.4387
d 45	0.88	0.83	1.00	0.17	
d 90	2.30	2.38	2.89	0.70	
d 135	4.51	4.18	4.33	0.69	
Birth	4.54	4.56	4.37	0.75	

[a-e] Denotes mean differences for the diet by day of gestation interaction ($P \leq 0.05$), within a variable.

[1] Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES and OVER, respectively. Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire the fetus(es). Lambs were collected within 24 h of birth from ewes allowed to undergo parturition (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per diet and day of gestation combination).

[2] LSMeans are reported.

[3] Organ weights are expressed as a percent of offspring BW.

[4] Adrenal gland weight is reported for sum of the pair of adrenal glands.

[5] nd = not detectable

[6] Kidney weight is reported for the sum of the pair of kidneys.

[7] Loin eye area was estimated using a plastic grid specific to pork and lamb (Iowa State University Extension and Outreach).

[8] Perirenal fat weight is reported for the combined weight of perirenal fat collected from around both kidneys.

Supplementary Table 1. Interaction of maternal diet by day of gestation by litter size for offspring BW and perirenal fat

	Treatment[1, 2]										
	CON			RES			OVER				
	Singleton	Twin	Triplet	Singleton	Twin	Triplet	Singleton	Twin	Triplet	SEM	*P*-value
BW, g											0.0011
d 45	10.55[a]	11.82[a]	9.80[a]	11.93[a]	9.05[a]	10.53[a]	10.90[a]	8.85[a]	9.48[a]	0.55	
d 90	585[a]	608[a]	607[a]	530[a]	626[a]	603[a]	607[a]	611[a]	657[a]	23	
d 135	4,535[abc]	5,267[cd]	3,793[a]	4,540[abc]	4,264[ab]	5,498[d]	5,072[cd]	4,497[b]	-	213	
Birth	7,257[a]	4,944[b]	3,883[c]	-	4,197[cf]	2,365[d]	5,805[e]	4,438[f]	4,233[cf]	234	
Perirenal fat,[3] %BW											0.0180
d 45	nd[4]	nd	nd	nd	nd	nd	nd	nd	nd	nd	
d 90	0.28[a]	0.52[a]	0.41[a]	0.37[a]	0.42[a]	0.41[a]	0.45[a]	0.42[a]	0.49[a]	0.079	
d 135	0.42[ab]	0.43[ab]	0.41[ab]	0.46[ab]	0.46[ab]	0.28[a]	0.58[b]	0.57[b]	-	0.082	
Birth	0.44[a]	0.36[a]	0.42[a]	-	0.47[a]	1.02[b]	0.26[a]	0.39[a]	0.33[a]	0.066	

[a-f] Denotes mean differences for the maternal diet by litter size interaction, within a day of gestation ($P \leq 0.05$).

[1] Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES and OVER, respectively. Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized a hysterectomy was performed to acquire the fetuses. Lambs were collected within 24 h of birth from ewes allowed to undergo parturition (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per treatment combination).

[2] LSMeans are reported.

[3] Perirenal fat is expressed as a percent of offspring BW and reported as the combined weight of perirenal fat collected from around

both kidneys.

[4] nd = not detectable.

Supplementary Table 2. Effect of maternal diet and day of gestation interaction on actual fetal organ weights

Fetal variable[1]	Treatment[2,3]			SEM	*P*-value
	CON	RES	OVER		Maternal diet by day of gestation
Adrenal gland wt,[4] g					0.6759
d 45	nd[5]	nd	nd	nd	
d 90	0.17	0.18	0.21	0.01	
d 135	0.58	0.47	0.53	0.02	
Birth	0.96	0.93	0.97	0.04	
Heart wt, g					0.0303
d 45	0.11 [a]	0.12[a]	0.12[a]	0.01	
d 90	5.06 [b]	4.70[b]	5.20[b]	0.13	
d 135	33.38[de]	25.18[c]	30.38[d]	1.22	
Birth	36.79 [e]	31.68[d]	36.57[e]	1.13	
Kidney wt,[6] g					0.0088
d 45	0.07 [a]	0.07 [a]	0.09 [a]	0.01	
d 90	5.84 [b]	5.73 [b]	6.19 [b]	0.14	
d 135	26.16[de]	21.11 [c]	27.48 [e]	0.87	
Birth	27.95 [e]	23.13[dc]	25.28[de]	0.80	
Liver wt, g					
d 45	0.76[a]	0.78[a]	0.60[a]	0.03	0.0030
d 90	33.55[b]	32.75[b]	36.87[b]	0.84	
d 135	136.43[d]	91.04[c]	129.95[d]	6.54	
Birth	105.65[c]	92.68[c]	103.84[c]	3.33	
Perirenal fat wt,[7] g					
d 45	nd	nd	nd	nd	0.1089
d 90	2.68	2.41	2.90	0.11	
d 135	20.90	18.60	26.48	0.89	
Birth	19.14	20.22	16.10	0.94	

[1] Organ weights are expressed as actual weights.

[2] Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES and OVER, respectively. Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized, and a hysterectomy was performed to acquire the fetus(es). Lambs were collected within 24 h of birth from ewes allowed to undergo parturition (n = 10 to 15 fetuses or lambs from five to seven ewes per diet and day of gestation combination).

[3] LSMeans are reported.

[4] Adrenal gland weight is reported for the sum of the weight of pair of adrenal glands.

[5] nd = not detectable

[6] Kidney weight is reported for the sum of weight of pair of kidneys.

[7] Perirenal fat weight is reported for the sum of weight of fat collected from around both kidneys.

CHAPTER 3: UNDERSTANDING PRENATAL MYOGENESIS AND EFFECTS OF POOR MATERNAL NUTRITION DURING GESTATION ON MUSCLE DEVELOPMENT IN FETAL SHEEP

Introduction

The fetal origins of disease hypothesis proposes that important physiological parameters can be reset by environmental influences during gestation and these changes can have significant impacts on the development, health and well-being outcomes for an individual ranging from infancy to adulthood (Barker, 1998; Barker and Hales, 2001). These changes include alterations to growth and development of tissues, body composition, metabolism, and expression of genes and proteins (Godfrey and Barker, 2001; Wu et al., 2006; Godfrey et al., 2010; Ford and Long, 2011; Varadinova et al., 2015). One of the potential mechanisms that is driving these changes is fetal programming of offspring, which is defined as the process whereby a stimulus or insult at a critical period of development has lasting or lifelong effects on the life of an organism (Nesterenko and Aly, 2009; Varadinova et al., 2015). One of the most important factors leading to fetal programming is poor maternal nutrition encountered by the developing offspring during gestation (Vonnahme, 2007; Yates et al., 2012). Poor maternal nutrition is defined as an alteration to macronutrient or micronutrient during gestation and under- and over-nutrition are the two most common forms encountered in livestock industry (Wu et al., 2006). Multiple tissues and organs such as liver, kidney, bone, skeletal muscle and adipose are affected by poor maternal nutrition during gestation (Fiorotto et al., 1995; Kind et al., 2005; Hyatt et al., 2008; Caton et al., 2009b; Hoffman et al., 2014; Jousse et al., 2014; Reed et al., 2014; Wood-Bradley et al., 2015).

Of particular interest is muscle tissue since it comprises a major portion of the body in Eutherian mammals, and plays a crucial role in locomotion and maintaining the metabolic

homeostasis in the body (Frontera and Ochala, 2015). In addition, muscle is the major tissue in meat and the quantity and quality of meat is dependent on the properties of muscle (Du et al., 2010b; Du et al., 2015). Therefore, optimum development of muscle is important for the maintaining the overall health of the organism and optimum production in meat producing animals (Oksbjerg et al., 2004; Du et al., 2015). Since a major portion of Eutherian myogenesis is completed during the prenatal development within the uterus, it is essential to understand the normal prenatal development of muscle and factors affecting the prenatal myogenesis (Bentzinger et al., 2012; Dumont et al., 2015). Studies done in multiple species have reported that poor maternal nutrition can affect muscle mass, fiber composition, cross-sectional area, connective tissue content and adiposity (McCoard et al., 2000; Bee, 2004; Quigley et al., 2005; Huang et al., 2010; Reed et al., 2014; Du et al., 2015; Hoffman et al., 2016b). In sheep, both under- and over-nutrition resulted in alterations of structural, physiologic and anatomical characters of muscle fibers and gene expression in muscles postnatally (Zhu et al., 2004; Tong et al., 2009; Reed et al., 2014). Studies have reported increase in fiber CSA and gene expression of muscle as early as 24 hours within birth of the lambs (Reed et al., 2014; Hoffman et al., 2016b). Only a few studies have been done using fetal models to understand the impact of maternal nutrition in sheep (Osgerby et al., 2002; Tong et al., 2009; Yan et al., 2013a; Penagaricano et al., 2014). S

A study done using ovine fetal model of maternal under-nutrition in sheep has previously reported that the muscle weight of semitendinosus muscle is reduced in offspring from underfed ewes in comparison with those from control fed ewes (Osgerby et al., 2002). Studies done using ovine fetal models of maternal over-nutrition have reported altered gene expression in fetal longissimus dorsi muscle at 75 days of gestation (Tong et al., 2009). This study reported changes

in myogenic markers including *MyoD, myogenin,* and *desmin* contents were reduced in OB compared with C fetal semitendenosus, indicating the downregulation of myogenesis. These studies reported the changes in muscle development at specific time points in the prenatal life of offspring because of exposure to either under- or over-feeding during gestation. In addition, the potential genetic mechanism behind the impacts of under and over-feeding on myogenesis may be different (Hoffman et al., 2016b).

Although studies have looked into the molecular mechanisms behind fetal skeletal muscle development, further studies are required to understand and validate these studies (Francis-West et al., 2003; Bentzinger et al., 2012; Xu et al., 2014; Dumont et al., 2015). Therefore, studies looking at the impacts of maternal under and over-nutrition, within the same model, on different stages of muscle development during gestation are warranted for a detailed and comprehensive understanding of normal muscle development and the impacts of maternal diet on muscle development. We hypothesized that poor maternal nutrition would alter CSA of muscle fibers and global gene expression of factors involved in the prenatal development and function of muscle tissue of lambs. Additionally, this study will contribute to understanding the changes in expression of genes contributing to muscle development and functioning during critical phases of myogenesis.

Materials and Methods

Animals

All animal procedures were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee (A13-059).

Eighty Western White-faced multiparous ewes were bred and estrus synchronized as previously described (Jones et al., 2016; Pillai et al., 2016). Briefly, a controlled intravaginal

drug release device (CIDR; Easi-Breed CIDR Sheep Insert, Zoetis, Florham, NJ) was inserted intravaginally into each ewe, removed after 12 days and then ewes received a single injection of $PGF_{2\alpha}$ i.m. [Lutalyse, 5 mg/mL; Zoetis; (Knights et al., 2001)]. Ewes group housed with one of four related Dorset rams were confirmed pregnant by trans-abdominal ultrasound on d 28.5 ± 0.4 (Jones et al., 2016) and were moved to individual pens twenty days after mating. Ewes were individually fed until the end of experiment and were randomly assigned into a 3 x 4 factorial arrangement of treatment structure at day 30 of gestation with main effects of diet (3) and time point of gestation (4). Pregnant ewes were transitioned onto a control (100% NRC requirements for TDN), restricted-fed (60% NRC requirements), or over-fed (140% NRC requirements) diet at d 31 ± 1.3 of gestation and remained on their respective diets until necropsy as described previously in Pillai et al. (2016). At day 45, day 90 or day 135 of gestation, ewes were weighed and euthanized with an i.v. injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ) containing 390 mg/mL sodium pentobarbital and 50 mg/mL phenytoin based on BW, followed by exsanguination as previously described (Reed et al., 2014). Subsequently, a hysterotomy was performed to remove the uterus and fetuses for tissue collection. A fourth group of ewes was allowed to undergo parturition. The fetuses and lambs from the Control (CON), Restricted-fed (RES) and Over-fed (OVER) ewes were collected at these time points, and tissue samples from these offspring were used for further analysis.

Sample collection

To perform cross-sectional area analysis offspring, longissimus dorsi (LM), semitendinosus (STN), and triceps brachii (TB) muscles were excised, weighed, and frozen in optimal cutting medium (OCT). Muscle samples from the midpoint of the left longissimus dorsi from 33 male offspring were used for the transcriptome analysis. Samples for the RNA-seq

analysis were immediately snap frozen in liquid nitrogen, transported to the laboratory and stored at -80 degree Celsius. 33 samples (n = 11, CON = 4, OVER = 3, RES = 4) day 135 (n = 11, CON = 4, OVER = 3, RES = 4) and birth (n = 11, CON = 4, OVER = 4, RES = 3) were used for RNA-seq analysis.

Immunohistochemistry

To determine the muscle fiber cross-sectional area (CSA), 10 µm muscle sections were collected using a Microm HM 525 (Thermo scientific, Waltham, MA). Sections were circled using PAP pen and then were dipped for 20 minutes in 4% paraformaldehyde in PBS. These sections were then washed three times with PBS and blocked for 20 minutes in 5% horse serum, 0.2% Triton-X100 in PBS. Subsequently, sections were placed in blocking solution containing wheat germ agglutinin AlexaFluor 594 (Invitrogen – 1:50 dilution) overnight at 4°C in a humidified chamber. This was followed by washing with PBS 3 times, and a coverslip was placed on top of sections with 1:9 Phosphate buffered saline: glycerol. Images (n = 5 per offspring) of muscle cross sections were taken at 20X magnification, and CSA was determined using ImageJ software. All data were analyzed using PROC MIXED (SAS Institute Inc, Cary, NC; version 9.4). CSA data were analyzed as a 3 x 4 factorial arrangement of treatments of diet (3) and day of gestation (4). Significance was considered if $P \leq 0.005$ and data is presented as LSMEANS ± SEM

Library preparation and sequencing

RNA extraction was performed as described (Reed et al., 2014; Hoffman et al., 2016b). A total of 50 ng of RNA from each sample was used to prepare sequencing libraries following Illumina's mRNASeq protocol. Libraries were sequenced with Illumina's NextSeq500 at the Center for Genomic Innovations, University of Connecticut, Storrs. The 33 libraries were

barcoded, multiplexed, and then sequenced.

Data Analysis

Quality control (QC) was performed using Sickle (Joshi and Fass, 2011) to eliminate sequences that were ≤ 35 bp in length and had a Phred score ≤ 35. Sequences were mapped to the Ovis aries (Oar_V.4.1) reference genomes using the STAR aligner (Reference for STAR), and splice junction was defined using NCBI GFF files for Ovis aries (Oar_V.4.1). Htseq count package was used to count the abundance of aligned reads (Anders et al., 2015). Differential gene expression was then determined using DESeq package in R-studio. Genes were considered to be differentially expressed when $P \leq 0.00005$ and the corresponding false discovery rate–corrected q-value was ≤ 0.05. Functional classifications, gene ontology, and gene enrichment analysis were performed on differentially expressed genes for day 90 vs day 135, day 90 vs day birth and day 135 vs birth comparisons that were identified via the Gene Ontology package in R-Studio/PANTHER classification system (Mi et al., 2013) using default parameters.

Results

Cross-sectional area

No significant interactions of diet by day of gestation were observed for fiber CSA of LM, STN, and TB ($P \geq 0.28$; Table 1). There was no main effect of poor maternal nutrition on the CSA of muscle fibers (Table 2). However, there was a significant main effect of time on the fiber CSA of LM, STN, and TB ($P \leq 0.0001$; Table 3). The average fiber CSA of LM decreased 46 % from day 45 to day 90 ($P \leq 0.004$; Table 3). In the case of STN and TB, there was no decrease in the CSA of muscle fibers from day 45 to day 90 ($P \geq 0.14$; Table 1). There was an increase of 152 %, 225% and 468 % in LM, STN and TB muscle fiber CSA respectively from day 90 to day 135 ($P \leq 0.0001$; Table 3). An increase of 15 % and 52 % was observed in LM and

STN fiber CSA from day 135 to Birth ($P \leq 0.0001$; Table 3). However, the CSA of the muscle fibers decreased 22 % in TB in comparison with CON ($P \leq 0.01$; Table 3).

Sequencing Output

The average number of raw reads were 26,731,710 for CON, 25,392,547 for RES, and 26,072,272 for OVER. The average number of short reads used for analysis after quality control and trimming were 22,536,680 for CON, 21,693,193 for RES, and 22,056,024 for OVER. Of the post-QC reads 84%, 85% and 85% mapped to the *Ovis aries* reference in case of CON, RES, and OVER, respectively. Htseq-count identified data for 27,721 genomic regions. Genes that had zero counts in all samples and genes with low coefficient, or high coefficient of variation were dropped from further analysis. Htseq-count data for the remaining 22,179 genes were analyzed using the DESeq2 package.

Differential gene expression

No significant interactions of diet by day of gestation were observed for the differential gene expression ($q \geq 0.22$). Main effect of diet and time was observed for number of genes ($q \leq 0.05$). Five downregulated, and two upregulated genes were identified when in the RES vs CON group ($q \leq 0.05$, Table 4). The downregulated genes include *Elongation factor 1-β pseudogene, Endoplasmic reticulum to nucleus signaling 2, Tripartite motif-containing protein 9,* and *Kinesin Family Member 20A* ($q \leq 0.05$, Table 5). The upregulated genes were *C-C motif chemokine 2 ligand* and *Pentraxin-related protein* ($q \leq 0.05$, Table 5). When the OVER vs CON comparison was made 9 downregulated, and 3 upregulated genes were identified ($q \leq 0.05$, Table 4). The downregulated genes were *Histone Deacetylase 10, Tripartite motif-containing protein 9, Transmembrane Channel Like 2, SH2 Domain Containing 6, Transmembrane Channel Like 8, Uncharacterized protein C9orf9homolog, Histone H1.3,* and *Serine/Threonine/Tyrosine*

Interacting Like 1($q \leq 0.05$, Table 5). *Craniofacial development protein pseudogene, Pappalysin-1and Elongation factor 1β pseudogene* were identified to be upregulated in OVER vs CON comparison ($q \leq 0.05$, Table 5). Two genes were upregulated, and 2 were downregulated in the OVER vs RES comparison ($q \leq 0.05$, Table 4). The genes that were downregulated were *Sterile Alpha Motif Domain Containing 14* and *Protein tyrosine phosphatase, receptor type Z1* and the genes that were upregulated were *Endoplasmic reticulum to nucleus signaling* 2 and *Elongation factor 1β pseudogene* ($q \leq 0.05$, Table 5).

In the day 90 vs day 135 day comparison 172 genes were downregulated and 137 genes were identified to be upregulated, whereas in the day 90 vs birth comparison 2378 genes were downregulated and 693 genes were upregulated ($q \leq 0.05$, Table 4). In the birth vs day 135 comparison 291 genes were identified to be downregulated and 80 genes were upregulated ($q \leq 0.05$, Table 4). The top 15 annotated and characterized genes with greatest fold change (down regulated and upregulated genes) from birth vs day 90, day 135 vs day 90 and birth vs day 135 are listed in Table 6, Table 7 and Table 8, respectively.

Gene list analysis

Gene list analysis did not identify any classification categories that were affected by maternal diet ($q > 0.05$). For gene classifications, 29.6% and 28.9% of the differentially expressed genes in birth vs day 90 were classified into the subcategories of binding (GO: 0005488) and catalytic activity (GO:0003824) respectively for the main categories of molecular function (Table 9). Additionally, for the main classification of biological processes, 44.50% of genes identified were categorized into the subcategory of cellular processes (GO: 0009987) and 32.70 % were classified into metabolic processes (GO: 0008152; Table 9). For cellular component for birth vs day 90, 20.3% and 12.4% belonged to the category of cell part and

organelle respectively and the genes were classified into 23 protein classes (Table 9).

In day 90 vs day 135, 27.50% of differentially expressed genes identified in the category of molecular function belonged to subcategory of binding (GO: 0005488) and 25.8% to catalytic activity (GO: 0003824; Table 10). For the main category of biological processes, 43.7 % of differentially expressed genes were classified into the subcategory of cellular processes (GO: 0008152) and 25.8% to the category of metabolic process (Table 10). For protein classifications, day 90 vs day 135 differentially expressed genes were subcategorized into 23 groups with nucleic acid binding ((PC00171); 10.9%) and signaling molecule ((PC00207); 8.3%). being the predominant groups (Table 10).

For gene classifications, 30.20% and 29.50% of differentially expressed genes in day 135 vs birth were classified into the subcategories of binding (GO: 0005488) and catalytic activity (GO:0003824) for the main categories of molecular function (Table 11). For the main category of biological processes 48.3% and 34.6% belonged to the subcategories of cellular process (GO:0009987) and metabolic process (GO:0008152), respectively (Table 11). The main category of cellular component had genes belonging to 8 subcategories of which cell part (GO:0044464) and organelle (GO:0043226) had the predominant number of genes (Table 11). The man category of protein class had genes belonging to 21 subcategories for birth vs day 135 of which the most number of genes were in the subcategories nucleic acid binding ((PC00171);13.1%) and enzyme modulator ((PC00095);8.1%, Table 11).

Table 1. Effect of maternal diet and day of gestation on cross-sectional area of muscle fibers

	Treatment[1, 2]				*P*-value
Muscle	CON	RES	OVER	SEM	Maternal diet by day of gestation
Longissimus dorsi					0.28
d 45	227.86	170.84	175.97	15.87	
d 90	102.20	101.37	104.71	15.80	
d 135	274.49	247.49	257.43	16.32	
Birth	291.92	283.53	321.94	16.42	
Semitendinosis					0.61
d 45	143.40	142.75	132.96	33.26	
d 90	110.41	98.11	115.56	32.58	
d 135	350.61	325.15	378.42	32.93	
Birth	580.32	470.21	561.04	33.62	
Triceps brachii					0.57
d 45	211.73	187.25	181.34	90.79	
d 90	157.22	141.13	137.32	91.71	
d 135	931.15	645.96	899.27	91.20	
Birth	625.23	537.35	751.49	94.43	

[1]Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES and OVER, respectively. Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins, and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized and a hysterotomy was performed to acquire the fetus(es). Lambs were collected within 24 h of birth (birth) from ewes allowed to undergo parturition (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per diet per day of gestation).

[2] LSMeans are reported.

Table 2. Main effect of maternal diet on cross-sectional area of muscle fibers

	Treatment[1, 2]				*P*-value
Muscle	CON	RES	OVER	SEM	Maternal diet
Longissimus dorsi	224.12	200.81	215.01	26.43	0.18
Semitendinosus	296.19	259.06	296.99	17.20	0.12
Triceps brachii	481.33	377.92	492.36	11.76	0.15

[1]Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES and OVER, respectively. Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins, and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized and a hysterotomy was performed to acquire the fetus(es). Lambs were collected within 24 h of birth (birth) from ewes allowed to undergo parturition (n = 40-50 fetuses or lambs from 5 to 7 ewes per diet).

[2] LSMeans are reported.

Table 3. Main effect of time on CSA of muscle fibers

	Timepoints[1, 2, 3]					*P*-value
Muscle	d 45	d 90	d 135	birth	SEM	Timepoint
Longissimus dorsi	191.56[a]	102.76[b]	259.80[c]	299.13[d]	9.31	0.0001
Semitendinosis	139.71[a]	108.03[a]	351.39[b]	537.19[c]	18.90	0.0001
Triceps brachii	193.44[a]	145.22[a]	825.46[b]	638.02[c]	53.43	0.0001

[a-d] Denotes mean differences for the day of gestation ($P \leq 0.05$) for each variable.

[1]Offspring from ewes fed a control (100% NRC), restricted (60% NRC), or over-fed (140% NRC). Ewe diet was based on the NRC TDN requirement for pregnant ewes bearing twins, and began at d 30 of gestation. At d 45, 90 or 135 of gestation, ewes were euthanized and a hysterotomy was performed to acquire the fetus(es). Lambs were collected within 24 h of birth (birth) from ewes allowed to undergo parturition (n = 35-40 fetuses or lambs per diet per day of gestation).

[2] LSMeans are reported.

[3] Values are expressed as averages of all values within a treatment at all the timepoints

Table 4. Effect of maternal diet[1] and day of gestation on gene expression of muscles[2]

Main effect	Comparison	Down-regulated	Up-regulated
Diet	RES vs CON	5	2
Diet	OVER vs CON	9	3
Diet	OVER vs RES	2	2
Time	day 90 to day 135	172	137
Time	day 90 to birth	2378	693
Time	day 135 to birth	291	80

[1]Lambs born to control-fed ewes (CON), born to restricted-fed ewes (RES), and lambs born to over-fed ewes (OVER).

[2]Longissimus dorsi muscles were collected from fetus ewes that were euthanized at day 90, day 135 of gestation or lambs within 24 of birth (birth) from ewes that were underwent the process of natural parturition.

Table 5. Differential gene expression treatment comparisons

Gene name	Description	Log fold change[1]
	RES vs CON[2]	
Elongation factor 1-beta pseudogene	Elongation factor	-24.27
Endoplasmic reticulum to nucleus signaling 2 (ERN2)	Cell signaling	-22.41
Tripartite motif-containing protein 9 (TRIM9)	Proteasomal degradation	-8.55
Kinesin Family Member 20A (KIF20A)	Mitosis and cellular proliferation	-6.86
LOC101103869	Unknown	-5.59
C-C motif chemokine 2 ligand (CCL2)	Monocyte chemotaxis	2.81
Pentraxin-related protein (PTX3)	Primary inflammatory signaling	2.70
	OVER vs CON	
Tripartite motif-containing protein 9 (TRIM9)	Proteasomal degradation	-7.25
Transmembrane Channel Like 2 (TMC2)	Ion transport	-6.18
SH2 Domain Containing 6 (SH2D6)	Intracellular signaling	-5.46
Transmembrane Channel Like 8 (TMC8)	Ion Transport	-5.40
Uncharacterized protein C9orf9homolog	Ion binding	-5.01
Histone H1.3 (H1.3)	Protein components of chromatin	-4.12
LOC106991095	Uncharacterized	-4.96
Serine/Threonine/Tyrosine Interacting Like 1	Pseudo phosphate	-3.82
Histone Deacetylase 10	Histone modification	-3.54
Craniofacial development protein pseudogene	Development	2.17
Pappalysin-1	Cleaves IGFBP4	3.01
Elongation factor 1-beta pseudogene	Pseudogene	19.01
	OVER vs RES	
Sterile Alpha Motif Domain Containing 14	Tumor suppressor	-6.28
Protein tyrosine phosphatase, receptor type Z1	Protein tyrosine phosphatase family	-5.33
Endoplasmic reticulum to nucleus signaling 2	Cell signaling	19.27
Elongation factor 1-beta pseudogene	Pseudogene	43.28

[1] Data are presented as log fold change from comparing the first treatment vs second treatment (CON vs RES, OVER vs CON and OVER vs CON

[2] Lambs born to control-fed ewes (CON), born to restricted-fed ewes (RES), and lambs born to over-fed ewes (OVER).

Table 6. Differentially expressed genes on day 90 to birth [1,2] (Top 15 hits based on log fold change)

Gene name	Description	Log fold change
	Downregulated	
microRNA 433 (MIR433)	Development and metabolism	-25.69
microRNA 431 (MIR431)	Regeneration of skeletal muscle	-24.68
microRNA 136 (MIR136)	Cell migration	-17.52
Periostin (POSTN)	Development and Regeneration	-10.12
Myosin light chain 4 (MYL4)	Embryonic muscle development	-8.57
Marker of proliferation Ki-67 (MKI67)	Cell proliferation	-8.43
Topoisomerase (DNA) II alpha (TOP2A)	Transcription	-7.67
Fras1 related extracellular matrix protein 1 (FREM1)	Epidermal differentiation	-7.38
Paternally expressed 10 (PEG 10)	Development	-7.38
Branched chain amino acid transaminase 1 (BCAT1)	Amino acid metabolism	-7.08
Ribonucleotide reductase regulatory subunit M 2 (RRM2)	Deoxyribonucleotides synthesis	-7.31
Kinesin family member 14 (KIF14)	Cell multiplication	-7.04
Solute Carrier Family 15 Member 2 (SLC15A2)	Peptide transport	-6.97
Collagen Type XXI Alpha 1 Chain (COL21A1)	Collagen synthesis	-6.83
Forkhead box protein M1 (FOXM1)	Cell cycle progression	-6.81
	Upregulated	
Glutathione S-transferase A4-like (GSTA4)	Cellular defense	19.59
Ring finger protein 183 (RNF183)	DNA repair	9.28
Muscarinic acetylcholine receptor (CHRM1)	Regulation of adenyl cyclase	9.00
Pyruvate dehydrogenase kinase isozyme 4 (PDK4)	Metabolism	8.39
Retinal degeneration 3 (RD3)	Protein binding	8.15
Solute Carrier Family 7 Member 8 (SL7A8)	Amino acid transport	7.99
Zymogen granule protein 16 (ZG16)	Protein Trafficking	7.92
Forkhead Box N4 (FOXN4)	Development	7.83
Armadillo Repeat Containing 12 (ARMC12)	Nuclear localization	7.76
Solute Carrier Family 14 Member 2 (SLC14A2)	Urea transporter	7.75
Receptor Transporter Protein 3 (RTP3)	Protein binding	7.74
Spi-B Transcription Factor (SPIB)	Transcription activity	7.04
Achaete-Scute-Like Protein 4 (ASCL4)	Differentiation, Protein binding	7.05
Lactase	Lactate metabolism	6.72

FOS Like 1, AP-1 Transcription Factor Subunit	Cell proliferation, Transcription	6.61

[1] Data are presented as log fold change from comparing the first treatment vs second treatment

[2]Longissimus dorsi muscles were collected from fetus ewes that were euthanized at day 90, day 135 of gestation or lambs (birth) from ewes that underwent the process of natural parturition.

Table 7. Differentially expressed genes from day 90 to day 135[1,2] (Top 15 hits based on log fold change)

Gene name	Description	Log fold change[1]
	Downregulated	
Solute Carrier Family 15 Member 2 (SLC15A2)	Peptide transport	-6.14
Zinc Finger CCHC-Type Containing 12 (ZCCHC12)	Transcriptional coactivator	-5.76
Palmitoleoyl-Protein Carboxylesterase (NOTUM)	Lipid regulation	-5.66
IQ Motif Containing G (IQCG)	Calcium level regulation	-5.54
Proline Rich 32 (PRR32)	Transcription	-5.23
Branched chain amino acid transaminase 1 (BCAT1)	Amino acid metabolism	-5.10
Cerebellin 2 Precursor (CBLN2)	Synapse formation	-5.06
GABA(A) Receptor Subunit Gamma-1	Neurotransmission	-5.02
Potassium Voltage-Gated Channel Subfamily H Member 6 (KCNH6)	Regulation of transmission	-4.94
Interleukin 17B (IL17B)	Regulation of inflammation	-4.82
Muscarinic acetylcholine receptor (CHRM2)	Regulation of adenyl cyclase	-4.76
Fibroblast Growth Factor 14 (FGF14)	Tissue Development	-4.72
F-Box and Leucine-Rich Repeat Protein 21 (FBXL21)	Protein Ubiquitination	-4.66
Neuronal Cell Adhesion Molecule (NRCAM)	Cell adhesion protein	-4.48
	Upregulated	
Glutathione S-transferase A4-like (GSTA4)	Cellular defense	20.55
60S Ribosomal Protein (RPL30)	Protein synthesis, RNA binding	8.65
Zymogen granule protein 16 (ZG16)	Protein Trafficking	7.93
Aldehyde Dehydrogenase 1 Family Member L1 (ALDH1L1)	Metabolism, Catalytic activity	7.46
Myosin Heavy Chain 1 (MYH1)	Contractile protein	6.90
Immunoglobulin-Like and Fibronectin Type III domain Containing 1 (IGFN1)	Transcription factor	5.94
Hemoglobin subunit beta (HBB)	Component of hemoglobin	
Acyl-CoA Synthetase Medium-Chain Family Member 5 (ACSM5)	Fatty acid metabolism	5.85
Apolipoprotein L3-like (APOL3)	Cholesterol transport	5.80

Eukaryotic Elongation Factor 1 Alpha 2 (EEF1A2)	Translation	5.35
Corticotropin Releasing Hormone Receptor2 (CRHR2)	Corticotropin-releasing factor	5.35
Fructose-Bisphosphatase 2 (FBP2)	Glucose metabolism	4.84
Actinin Alpha 3 (ACTN3)	Crosslinking actin thin filaments.	4.77
Zinc Finger and BT Domain Containing 16 (ZBTB16)	Cell cycle progression and regulation	4.59
Glutathione S-Transferase Alpha 1	Glutathione transferase activity	4.55

[1] Data are presented as log fold change from comparing the first treatment vs second treatment

[2]Longissimus dorsi muscles were collected from fetus ewes that were euthanized at day 90, day 135 of gestation or lambs (birth) from ewes that were underwent the process of natural parturition.

Table 8. Differentially expressed genes birth vs day 135 (Top 15 hits based on log fold change)

Gene name	Description	Log fold change[1]
	Downregulated	
microRNA 433 (MIR433)	Development and metabolism	-22.77
microRNA 431 (MIR431)	Regeneration of skeletal muscle	-22.21
Marker of proliferation Ki-67 (MKI67)	Cell proliferation	-6.65
Periostin (POSTN)	Development and Regeneration	-5.57
Topoisomerase (DNA) II alpha (TOP2A)	Transcription	-5.32
Forkhead Box M1 (FoxM1)	Transcriptional and Cell proliferation	-4.92
ST6 N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5 (ST6GALNAC5)	Modulate cell interactions	-4.89
Kinesin Family Member 14 (KIF14)	Cell division, Microtubule formation	-4.86
Chordin Like 2 (CHRDL2)	Differentiation	-4.82
Holliday Junction Recognition Protein (HJURP)	Regulation of CENPA	-4.80
Centromere Protein A (CENPA)	Histone - Nucleosome structure	-4.76
Ribonucleotide Reductase M2 Polypeptide (RRM2)	Deoxyribonucleotides synthesis	-4.71
Proline Rich 11 (PRR11)	Cell cycle progression	-4.67
Sialyltransferase 2 (ST6GAL2)	Sialic acid metabolism	-4.63
	Upregulated	
Pyruvate dehydrogenase kinase isozyme 4 (PDK4)	Metabolism	7.36
Histone H1.4 (HIST1H1E)	Nucleosome component	6.92
Armadillo Repeat Containing 12 (ARMC12)	Transcription	5.76
Serine Dehydratase (SDS)	Metabolism of Serine	5.29
Tripartite Motif Containing 63 (TRIM63)	Myosin heavy chain degradation	4.80
Adenosine Monophosphate Deaminase 3(GREB1)	hydrolytic deamination of AMP	4.49
Malonyl-CoA Decarboxylase (MLYCD)	Fatty acid biosynthesis	3.88
Histone H1.3 (HIST1H1D)	Protein components of chromatin	3.50
Fatty Acid Binding Protein 3 (FABP3)	Fatty acid metabolism	3.12
Sestrin 1 (SESN1)	Regulation of proliferation	3.01
Histone Deacetylase 10 (HDAC10)	Histone modification	2.96
Ras Related Dexamethasone Induced (RASD1)	GTPase activity	2.84
Complement C5 (C5)	Innate immune system	2.80
CCAAT/Enhancer Binding Protein Epsilon (CEBP)	Adipocyte differentiation	2.77

[1] Data are presented as log fold change from comparing the first treatment vs second treatment

[2]Longissimus dorsi muscles were collected from fetus ewes that were euthanized at day 90, day 135 of gestation or lambs (birth) from ewes that underwent the process of natural parturition.

Table 9. Gene classification and ontology for day 90 vs birth

	Birth vs Day 90	
CLASSIFICATION	**No of genes**	**% of genes**
Molecular Function		
binding (GO:0005488)	682	29.6
catalytic activity (GO:0003824)	665	28.9
receptor activity (GO:0004872)	137	6.0
transporter activity (GO:0005215)	136	5.9
structural molecule activity (GO:0005198)	128	5.6
signal transducer activity (GO:0004871)	46	2.0
translation regulator activity (GO:0045182)	9	0.4
antioxidant activity (GO:0016209)	5	0.2
channel regulator activity (GO:0016247)	3	0.1
Biological Processes		
cellular process (GO:0009987)	1025	44.5
metabolic process (GO:0008152)	752	32.7
developmental process (GO:0032502)	291	12.6
response to stimulus (GO:0050896)	258	11.2
localization (GO:0051179)	252	10.9
Biological regulation	249	10.8
multicellular organismal process (GO:0032501)	225	9.8
biogenesis (GO:0071840)	199	8.6
immune system process (GO:0002376)	131	5.7
biological adhesion (GO:0022610)	90	3.9
reproduction (GO:0000003)	46	2.0
locomotion (GO:0040011)	37	1.6
Cellular Component		
cell part (GO:0044464)	467	20.3
organelle (GO:0043226)	285	12.4
membrane (GO:0016020)	220	9.6
macromolecular complex (GO:0032991)	126	5.5
extracellular region (GO:0005576)	111	4.8
extracellular matrix (GO:0031012)	45	2.0
cell junction (GO:0030054)	12	0.5
synapse (GO:0045202)	7	0.3
Protein Class		
nucleic acid binding (PC00171)	241	10.5
hydrolase (PC00121)	200	8.7

enzyme modulator (PC00095)	192	8.3
transferase (PC00220)	158	6.9
transcription factor (PC00218)	156	6.8
signaling molecule (PC00207)	152	6.6
receptor (PC00197)	149	6.0
cytoskeletal protein (PC00085)	132	5.7
transporter (PC00227)	128	5.6
extracellular matrix protein (PC00102)	75	3.3
oxidoreductase (PC00176)	72	3.1
cell adhesion molecule (PC00069)	71	3.1
calcium-binding protein (PC00060)	46	2.0
defense/immunity protein (PC00090)	42	1.8
transfer/carrier protein (PC00219)	38	1.7
ligase (PC00142)	32	1.4
structural protein (PC00211)	27	1.2
membrane traffic protein (PC00150)	26	1.1
cell junction protein (PC00070)	22	1.0
lyase (PC00144)	16	0.7
chaperone (PC00072)	12	0.5
isomerase (PC00135)	12	0.5
transmembrane receptor regulatory protein (PC00226)	7	0.3

Differentially expressed genes were classified into one of three major categories and correlating gene ontology classifications. Genes were also classified by protein class. Classification was performed using the Panther classification system. Percentages were determined by the number of input genes that corresponded with a given classification against the total number of genes hit.

Table 10. Gene classification and ontology for day 90 vs day 135

CLASSIFICATION	Day 135 vs Day 90	
	No of genes	**% of genes**
Molecular Function		
binding (GO:0005488)	63	27.5
catalytic activity (GO:0003824)	59	25.8
receptor activity (GO:0004872)	19	8.3
transporter activity (GO:0005215)	16	7.0
structural molecule activity (GO:0005198)	15	6.6
signal transducer activity (GO:0004871)	6	2.6
translation regulator activity (GO:0045182)	1	0.4
channel regulator activity (GO:0016247)	1	0.4
Biological Processes		
cellular process (GO:0009987)	100	43.7
metabolic process (GO:0008152)	59	25.8
developmental process (GO:0032502)	25	10.9
response to stimulus (GO:0050896)	27	11.8
localization (GO:0051179)	27	11.8
Biological regulation	25	10.9
multicellular organismal process (GO:0032501)	24	10.5
biogenesis (GO:0071840)	23	10.0
immune system process (GO:0002376)	11	4.8
biological adhesion (GO:0022610)	5	2.2
reproduction (GO:0000003)	4	1.7
locomotion (GO:0040011)	3	1.3
Cellular Component		
cell part (GO:0044464)	47	20.5
organelle (GO:0043226)	27	11.8
membrane (GO:0016020)	23	10.0
macromolecular complex (GO:0032991)	12	5.2
extracellular region (GO:0005576)	10	4.4
extracellular matrix (GO:0031012)	1	0.4
cell junction (GO:0030054)	2	0
Protein Class		
nucleic acid binding (PC00171)	25	10.9
signaling molecule (PC00207)	19	8.3
hydrolase (PC00121)	19	8.3
cytoskeletal protein (PC00085)	17	7.4

receptor (PC00197)	17	7.4
transporter (PC00227)	16	7.0
transferase (PC00220)	16	7.0
enzyme modulator (PC00095)	12	5.2
transcription factor (PC00218)	12	5.2
oxidoreductase (PC00176)	7	3.1
cell adhesion molecule (PC00069)	6	2.6
defense/immunity protein (PC00090)	6	2.6
extracellular matrix protein (PC00102)	5	2.2
structural protein (PC00211)	5	2.2
ligase (PC00142)	4	1.7
transfer/carrier protein (PC00219)	4	1.7
lyase (PC00144)	3	1.3
chaperone (PC00072)	3	1.3
transmembrane receptor regulatory(PC00226)	2	0.9
calcium-binding protein (PC00060)	2	0.9
membrane traffic protein (PC00150)	2	0.9
cell junction protein (PC00070)	1	0.4
isomerase (PC00135)	1	0.4

Differentially expressed genes were classified into one of three major categories and correlating gene ontology classifications. Genes were also classified by protein class. Classification was performed using the Panther classification system. Percentages were determined by the number of input genes that corresponded with a given classification against the total number of genes hit.

Table 11. Gene classification and ontology for day 135 vs birth

	birth vs day 135	
CLASSIFICATION	**No. of genes**	**% of genes**
Molecular Function		
binding (GO:0005488)	90	30.2
catalytic activity (GO:0003824)	88	29.5
structural molecule activity (GO:0005198)	21	7.0
receptor activity (GO:0004872)	18	6.0
transporter activity (GO:0005215)	11	3.7
signal transducer activity (GO:0004871)	8	2.7
translation regulator activity (GO:0045182)	2	0.7
antioxidant activity (GO:0016209)	1	0.3
Biological Processes		
cellular process (GO:0009987)	144	48.3
metabolic process (GO:0008152)	103	34.6
developmental process (GO:0032502)	39	13.1
response to stimulus (GO:0050896)	35	11.7
biological regulation (GO:0065007)	33	11.1
multicellular organismal process (GO:0032501)	28	9.4
biogenesis (GO:0071840)	26	8.7
localization (GO:0051179)	25	8.4
immune system process (GO:0002376)	17	5.7
biological adhesion (GO:0022610)	15	5.0
cellular process (GO:0009987)	144	48.3
metabolic process (GO:0008152)	103	34.6
Cellular Component		
cell part (GO:0044464)	73	24.5
organelle (GO:0043226)	47	15.8
macromolecular complex (GO:0032991)	25	8.4
membrane (GO:0016020)	24	8.1
extracellular region (GO:0005576)	11	3.7
extracellular matrix (GO:0031012)	8	2.7
cell junction (GO:0030054)	2	0.7
synapse (GO:0045202)	1	0.3
Protein Class		
nucleic acid binding (PC00171)	39	13.1
enzyme modulator (PC00095)	24	8.1
transferase (PC00220)	22	7.4

cytoskeletal protein (PC00085)	21	7.0
transcription factor (PC00218)	20	6.7
signaling molecule (PC00207)	19	6.4
hydrolase (PC00121)	19	6.4
receptor (PC00197)	18	6.0
transporter (PC00227)	13	4.4
cell adhesion molecule (PC00069)	10	3.4
extracellular matrix protein (PC00102)	9	3.0
oxidoreductase (PC00176)	6	2.0
calcium-binding protein (PC00060)	6	2.0
structural protein (PC00211)	6	2.0
ligase (PC00142)	5	1.7
defense/immunity protein (PC00090)	5	1.7
cell junction protein (PC00070)	5	1.7
transfer/carrier protein (PC00219)	3	1.0
isomerase (PC00135)	3	1.0
lyase (PC00144)	2	0.7
membrane traffic protein (PC00150)	2	0.7

Differentially expressed genes were classified into one of three major categories and correlating gene ontology classifications. Genes were also classified by protein class. Classification was performed using the Panther classification system. Percentages were determined by the number of input genes that corresponded with a given classification against the total number of genes hit.

Discussion

Multiple studies have demonstrated that maternal plane of nutrition during different stages of pregnancy can modify the fetal development of skeletal muscle (McCoard et al., 2000; Osgerby et al., 2002; Bee, 2004; Quigley et al., 2005; Tong et al., 2009; Yan et al., 2011; Yan et al., 2013a; Penagaricano et al., 2014; Raja et al., 2016). This, in turn, could have important implications in meat production and general health of the offspring (Oksbjerg et al., 2004; Varadinova et al., 2015; Du et al., 2015). The number of muscle fibers in eutherian mammals is determined by birth and therefore the period of prenatal growth is important from a muscle development perspective (Greenwood and Thompson, 2007; Du et al., 2011; Frontera and Ochala, 2015). Furthermore, muscle development in Eutherian mammals occurs in waves during specific stages of gestation is are referred to as critical stages of development (Bentzinger et al., 2012). Insults occurring to the offspring during these critical stages of muscle development can impact the development and function of muscle fibers. Prenatal muscle development is a tightly regulated by intrinsic factors that can be influenced by the external environment and in turn can impact the development of skeletal muscles (Francis-West et al., 2003; Bentzinger et al., 2012; Dumont et al., 2015). The absence of effect in the difference of CSA area at day 45 might be because the animals have been exposed to the poor maternal diet only for a very short duration of time before the muscles were evaluated.

Studies have reported that overfeeding of ewes can result in reduced CSA of muscle fibers at 75 days of gestation in offspring (Tong et al., 2009). Another model of nutrient restriction during the period around conception in sheep resulted in an increase in the CSA of secondary fibers at day 90 of gestation without changes in the overall CSA of muscle fibers and primary fibers (Sen et al., 2016). Therefore, the CSA of primary and secondary muscle fibers

need to be separately analyzed to completely understand the impacts of CSA on muscle fibers. This is important because fiber type specific alterations have been reported in multiple species postnatally as a result of exposure to poor maternal nutrition (Zhu et al., 2006; Reed et al., 2014; Confortim et al., 2015). The reduction in the CSA of muscle fibers observed at day 90 is due to the development of secondary myofibers from primary myofibers during mid gestation reported in the current literature (Zhu et al., 2004; Zhu et al., 2008; Tong et al., 2009; Du et al., 2011). The increase in the CSA from day 90 to day 135 and birth reported in muscle fiber is due to the late gestation hypertrophy observed in Eutherian mammals (Du et al., 2010b; 2011; 2015). This study is unique in its model because it is one of the few studies in sheep that traced the molecular and morphologic changes during muscle development during the critical phases of skeletal muscle development including primary myogenesis to secondary myogenesis and late term hypertrophy. In addition, this model also evaluated the impacts of maternal under- and over-nutrition on the stages of muscle development in the same experiment.

Skeletal muscle is a highly complex and heterogeneous tissue serving a multitude of functions in the organism (Frontera and Ochala, 2015). The process of myogenesis can be divided into distinct phases and each phase is tightly regulated by multiple molecular mechanisms (Bentzinger et al., 2012). Alterations to the diet can affect the expression of these factors and lead to alterations in the development and functioning of muscles. Endoplasmic reticulum (ER) to nucleus signaling 2 (*ERN2*) is a gene involved in the signaling of the ER stress in the cells (Kanekura et al., 2015). Certain level of ER stress is required for the protein accretion and therefore adequate muscle development in mammals (Nakanishi et al., 2005). The down regulation of these genes in RES compared with CON is suggests that the ER stress in the RES is reduced and this may be indicating the reduced protein accretion and muscle development in the

RES offspring. *TRIM 9* is a ubiquitin ligase involved in a neuronal function and is reported to be localized to the brain (Tanji et al., 2010). The identification of this gene in the muscle is being reported for the first time and is suggesting that this gene might have a role in neuronal development and nerve innervation in the muscle. Down regulation of these genes in the RES and OVER in comparison with the CON might indicate that neuronal development could be altered in the RES as a result of exposure to poor maternal nutrition.

Pentraxin (PTX3) 3 is associated with inflammation, insulin signaling, and considered as a biomarker of metabolic syndrome in humans (Miyaki et al., 2014; Kardas et al., 2015). Therefore, the upregulation of *PTX3* in the RES is indicative of altered metabolic and inflammatory status of the muscle. This may have an impact on the metabolic functions of the muscle and thereby the overall metabolic status of the offspring. *Chemokine (C-C motif) ligand 2* (*CCL2*) is associated with muscle adaptation, regeneration, injury and strength (Harmon et al., 2010). The upregulation of *CCL2* in the RES may be a adaptive response of the muscles to the inflammatory stress caused by restricted nutrition.

Regulation of gene expression without altering the primary DNA nucleotide sequence is the essence behind the concept of epigenetic regulation and epigenetic modification include histone modifications, and DNA methylation (Sincennes et al., 2016). Alterations of epigenetic regulation are known to be a potential mechanism for the impacts of poor maternal nutrition (Simmons, 2011; Feeney et al., 2014). Studies have demonstrated the epigenetic regulation of key myogenic regulatory factors and that HDAC are involved in the regulation of these myogenic regulatory factors (Feeney et al., 2014). Also, *HDAC10* is reported to be a repressor of microRNA let-7 g expression, which is important for adipogenesis (Sun et al., 2009; Li et al., 2015). Alterations to microRNA let-7 g have also been reported in skeletal muscle tissues of

offspring from obese mothers (Yan et al., 2013a). Therefore, HDAC might be exerting a role on adipogenesis in skeletal muscle through regulation of microRNA let-7 g. Also, the expression of *Histone H1.3* is which is important for condensation of nucleosome chains and maintenance of chromatin structure is also down regulated in the OVER compared with CON. Pappalysin-1 is a metalloproteinase that cleaves *IGF-binding protein 4* (*IGFBP4*). Knockout studies done on pappalysin using mice models have demonstrated that deletion of *Pappalysin-1* gene decreases bioavailability of *IGF-1* due to an increased abundance of *IGFBP-4* (Junnila et al., 2013). Therefore, the upregulation in the *Pappalysin-1* gene in OVER compared with CON may contribute to the alterations in the signaling though somatotropic axis and thereby the growth and development of the animals.

MicroRNAs are involved in the regulation of many cellular processes such as cell-fate determination, proliferation, differentiation and apoptosis, by controlling gene expression at the transcriptional or post-transcriptional level (Sokol, 2012). Their involvement in the development and functions of muscle have been studied in detail in recent years (Diniz and Wang, 2016; Simionescu-Bankston and Kumar, 2016). The downregulation of the *MIR433, MIR431* and *MIR136* is notable at birth compared with day 90 is interesting from a muscle development perspective. In addition, *MIR433* and *MIR431* are also down regulated at day 135 in comparison with birth. Of particular interest is *MIR431*, which is known to directly modulate and suppress the activity of *Pax7* transcription factors and promote muscle regeneration in mice in mice (Wu et al., 2015). In contrast to the findings in literature, *Pax* 7 did not respond to downregulation of *MIR431* by upregulating its expression at day 135 compared with birth, but was downregulated at birth in comparison with day 90. The downregulation in myogenic regulatory factor *MYF5* at birth is expected as these genes are known to play a role in the early commitment of myogenic

precursors to become myoblast. The downregulation of IGF1, myosin heavy chain isoform *MYH15*, collagen *COL21A1* and regulator of cell cycle progression *FOXM1* at birth in comparison with the day 90 may be due to the hypertrophy, protein accretion and fibrogenesis occurring in late gestation. Upregulation of *PDK4* and adenyl cyclase are indicating changes in metabolism from d 90 to birth and is also indicating alterations to metabolism.

The upregulation of *60S Ribosomal Protein* and *Zymogen granule protein 16* are indicative of increased protein synthesis and protein transportation, respectively (Bentzinger et al., 2012). Increased protein accretion is a characteristic of late term hypertrophy and therefore the upregulation of these genes may be to meet the demand of increased protein accretion at day 135 compared with day 90. The upregulation of the factors involved in *Fructose-Bisphosphatase 2*, *Aldehyde Dehydrogenase 1 Family Member L1* and *Acyl-CoA Synthetase Medium-Chain Family Member 5* may be indicative of increased carbohydrate metabolism and fatty acid metabolism in the muscle fibers in preparation for increased functioning of these muscles at birth to support locomotion.

The biological pathways that are affected the most over time (birth vs day 90, birth vs day 135 and day 135 vs day 90) are cellular process, metabolic process and developmental process in all three comparisons. This is in agreement with the literature that there is a shift in metabolic and development process in muscles from mid-gestation to late gestation and birth (McCoard et al., 2000; Zhu et al., 2008; Du et al., 2011; Yates et al., 2012; Bentzinger et al., 2012). The molecular functions that are impacted, including binding and catalytic activity which are also known to be altered during different stages of fetal muscle development (Du et al., 2010b; Bentzinger et al., 2012; Yates et al., 2012; Dumont et al., 2015). In all three-pathway analyses used, the protein class that was found to be greatly impacted was the nucleic acid

binding group. This is in agreement with the existing literature, which also reports tight regulation in the expression of genes and transcription factors during different stages of gestation (Zhu et al., 2006; Tong et al., 2009; Sokol, 2012; Bentzinger et al., 2012; Zhao et al., 2016).

Conclusion

In conclusion, this study showed that gene expression was altered in the fetal skeletal muscle of under- and over-fed ewes. Interestingly the changes in gene expression was different in the under- and over-fed in comparison with the controls. This agrees with previous study conducted on postnatal time points, with similar degrees of under- or over-feeding, and identified that even with similar phenotypic changes in the muscle, the potential gene expression alterations behind the changes could be different (Hoffman et al., 2016b). Furthermore, this study has given insights into the gene expression changes over time during critical points of prenatal ovine muscle development.

GENERAL DISCUSSION

With the increasing population around the world, there is increased demand for food, and this necessitates an increase in production efficiency. Production efficiency, in livestock production setting, is dependent on the optimum growth and development of offspring as well as maintenance of general health (Du et al., 2015). Multiple genetic and environmental factors have been identified that has a crucial role in animal performance in livestock species (Reynolds et al., 2010). One factor that has been of growing interest in recent years is fetal programming and factors that can potentially contribute to fetal programming (Barker, 1998; Godfrey and Barker, 2001; Almond and Currie, 2011). Fetal programming can be caused by multiple factors including the influence of intrauterine conditions in which the mammalian fetus develops (McMillen and Robinson, 2005; Wu et al., 2006; Godfrey et al., 2010; Varadinova et al., 2015). One of the most important factors that impacts the fetal growth and development is the nutrient supply that the fetus receives when it is developing in-utero (Redmer et al., 2004; Wu et al., 2006; Almond and Currie, 2011). Experimental studies using animal models and epidemiological data from human studies suggest that maternal diet has a significant impact on normal growth of the fetus in-utero and long-term health of the animals (Nesterenko and Aly, 2009; Reynolds et al., 2010; Meyer et al., 2010; Ge et al., 2013; Hoffman et al., 2014; Varadinova et al., 2015; Kleemann et al., 2015). In addition, studies completed in recent years have reported that many of these changes can be irreversible and can be transmitted across generations through epigenetic alterations (Simmons, 2011; Feeney et al., 2014).

Overall the aim of the research presented was to evaluate the impacts of poor maternal nutrition on offspring growth, development, and metabolism with special focus on muscle, bone,

and adipose tissues. The study also aimed at understanding the potential mechanisms behind the impacts observed in muscle, adipose and bone by understanding the changes in molecular mechanisms and alterations to the common progenitor stem cells- MSC.

Mesenchymal stem cells are key components of the bone marrow niche, which is responsive to hormonal and metabolic changes in the whole body and are the progenitor stem cells of bone, adipose and muscle tissues (Caplan, 2015). Although no changes were observed in bone development, changes to muscle and adipose tissue development, were observed in the study mentioned in Chapter 1, as a result of exposure to maternal nutrition (Reed et al., 2014). Other studies have also reported similar results with muscle, bone and fat as a result of exposure to maternal nutrition (Cooper et al., 1997; Wu et al., 2006; Zhu et al., 2006; Ford et al., 2007; Zhu et al., 2008; Tong et al., 2009; Long et al., 2010; Yan et al., 2011). Since MSC give rise to these tissues alterations in the function of MSC may be a potential mechanism behind the changes observed in muscle, adipose development, and metabolism. The reduction in proliferation is in agreement with current literature where restricted protein feeding in dams lead to a reduction in the proliferation of MSC of offspring in a rodent model (Oreffo et al., 2003). The reduction in proliferation may prevent optimum development of tissues, maintenance of these tissues in their adult life and healing during injury, since self-renewal of stem cell pools is important for development of tissues as well as maintenance and healing of the tissues in case of injury (Caplan and Hariri, 2015; Caplan, 2015; 2016). Differentiation and metabolism of MSC are correlated and therefore the alterations to metabolism can lead to changes in the differentiation ability of these cells into different lineages (Varum et al., 2011; Shum et al., 2016). This, in turn, can impact the development and function of tissues that arise from MSC such as bone, adipose, and muscle. There is evidence that factors, such as maternal diet, may

cause MSC to favor differentiation into one cell lineage versus another (adipose vs. bone; Devlin and Bouxsein, 2012). This diversion could be a potential mechanism by which poor maternal diet alters offspring growth and favors adiposity postnatally (Devlin and Bouxsein, 2012). The changes observed in the proliferation and metabolism observed were in controlled culture conditions, suggesting potential epigenetic modifications of MSC during development. The findings of reduced MSC function and metabolic activity suggest that maternal diet may program impaired offspring growth and metabolism through permanent modifications to offspring stem cells.

In the studies from Chapter 2, it is evident that liver and kidney organs are impacted by the impacts of poor maternal nutrition. Other studies have elucidated the alterations to the development of kidney and liver as a result of exposure to poor maternal nutrition (Hyatt et al., 2007; Caton et al., 2009b; Benz and Amann, 2010). Moreover, results from studies conducted and detailed in Chapter 3 and data from current literature proves that the development of muscle is altered as a result of exposure to poor maternal nutrition during gestation (Zhu et al., 2008; Tong et al., 2009; Reed et al., 2014; Hoffman et al., 2016b). The function of muscles, especially metabolism, is closely associated with liver metabolism (Kmiec, 2001; Georgieva et al., 2003; Meyer et al., 2004). The coordinated regulation of carbohydrate, lipid and amino acid metabolism between liver and muscle is essential to maintain the homeostasis in the body. For instance, coordination between liver, kidney, and muscle are also essential for maintaining post-prandial glucose homeostasis in the body (Meyer et al., 2002). The alterations to the functions of either liver or muscle can lead to dysregulation in the other, as in the case of type 2 diabetes (Meyer et al., 2004). Therefore, the changes observed in liver exacerbate the negative impacts on muscle and vice versa. Kidney is another tissue impacted by poor maternal nutrition as reported

in Chapter 2. Other studies that have evaluated the impacts of maternal diet on kidney development also have reported altered developmental patterns in kidney (Benz and Amann, 2010; Luyckx and Brenner, 2015). Kidney plays a major role in the excretion of byproducts from protein metabolism and degradation in the form of urea, uric acid, and creatinine. In addition, kidney plays an important role in retaining and regulating protein excretion and oncotic pressure in the body (Moritz and Wintour, 1999) and is, therefore, important for muscle development. In addition, the functions of kidney are closely related with the adequate development of bones, bone remodeling and bone resorption (Gal-Moscovici and Sprague, 2007). In essence, poor maternal diet impacts multiple offspring organ/organ systems, and since the functions of various organ systems are interdependent, the impacts of maternal diet on one organ/organ system has a potential impact on other organ/organ systems. Since the present study only evaluated the changes in organ weights, further functional and molecular studies are warranted to understand potential mechanism behind these changes.

As previously reported by our laboratory, poor maternal nutrition altered the CSA of muscles as early as within 24 hours of birth (Reed et al., 2014; Raja et al., 2016) . The study focusing on the postnatal time points found that alterations in gene expression of key myogenic regulatory factors in muscles and satellites cells (Reed et al., 2014; Raja et al., 2016). Since these changes were observed at birth, the next logical step was to determine the timing during prenatal development during which these changes occur. Therefore, effects of poor maternal nutrition on muscle development were analyzed in the in-vivo studies mentioned earlier in Chapter 1 and Chapter3. Although changes in the CSA of the offspring were not observed in the current study described in Chapter 3, several changes in gene expression were observed. The changes in expression of genes involved in epigenetic regulation such as *HDAC10* and *H1.3* suggests that

the epigenetic regulation in these cells might be altered. Studies have reported epigenetic alterations in the skeletal muscle as a result of exposure to poor maternal nutrition (Simmons, 2011; Lee, 2015; Sincennes et al., 2016). Therefore, further studies evaluating the epigenetic alterations in muscle arising as a result of maternal diet is warranted. Genes involved in the development and metabolism of muscles were also altered suggesting possible changes in the morphology, metabolism and function of muscle fibers in later life of the offspring. The impacts can result in negative impacts on the general health as well as quality and quantity of meat production in these animals.

This study also tracked the prenatal muscle development of ovine fetus over time. The changes in the CSA of muscle fibers over time followed the predicted pattern of prenatal myogenesis in sheep based on current literature. Moreover, the research also provides us with valuable information about the molecular regulation of prenatal myogenesis in sheep. On performing pathway analysis, key biological process, molecular functions and cellular changes involved in muscle development were identified. The data from the study presented in Chapter3 is valuable in understanding the normal development of muscle during gestation as well as the impacts of poor maternal nutrition on muscle development. Previous studies conducted by us demonstrated that even with similar changes in CSA of muscle in under-fed and over-fed, the potential molecular mechanism behind these changes might be different (Reed et al., 2014; Hoffman et al., 2016b). The current study also has similar results, in which, although there is no change in CSA of muscle fibers as a result of exposure to poor maternal nutrition, the molecular mechanism as evaluated by gene expression is not similar.

The balance in the development of adipose, bone and muscle are essential for the optimum carcass quality in meat production. Excess or little of fat content affects the quality and

quantity of meat. The increase in renal fat content observed in offspring exposed to poor maternal nutrition may suggest increased adiposity in the offspring which is consistent with current literature that have also reported increased adiposity in offspring exposed to poor maternal nutrition (Fiorotto et al., 1995; Kind et al., 2005; Louey et al., 2005; Ford et al., 2009). Increased adiposity can cause increased secretion of inflammatory cytokines such as TNF-α, decrease expression of GLUT-4 glucose transporters and affect the insulin sensitivity and thereby functioning of muscles (Costello et al., 2013; Yan et al., 2013a). Furthermore, as stated earlier, the development of excess adipose might be at the cost of bone mass in later life (Devlin and Bouxsein, 2012). This reduction in bone might affect the skeletal framework, which indirectly can affect the function and morphology of muscles.

In summary, poor maternal nutrition can alter several aspects of offspring growth, development and metabolism including the gene expression of muscles, stem cell function and metabolism and development of organs. The two separate studies conducted to evaluate impacts of poor maternal nutrition during prenatal and early postnatal time points identified changes to the growth and composition of the tissues as well as potential mechanisms behind these changes. The potential mechanisms identified in these studies were alterations to stem cells and changes in gene expression, both of which are suggesting altered fetal programming. Additional studies are warranted to confirm whether the changes observed in the gene expression of muscles are also reflected at the level of protein expression. Since the role of epigenetics as a potential mechanism behind the permanent and transgenerational transmission of poor maternal nutrition is becoming more evident, studies to elucidate the epigenetic alteration in muscle and MSC are necessary. Multiple epidemiological and animal studies, including the two in-vivo experiments studies that have been discussed in this dissertation have clearly demonstrated that poor maternal nutrition

affects various aspects of offspring, growth, development and metabolism. Therefore, intervention studies to mitigate the negative impacts of poor maternal diet should also be considered.

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